Thesis title: Developing an efficient process for the production of retinal progenitor cells (RPCs) from human pluripotent stem cells using lowered oxygen tension

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Abstract

The efficient differentiation of retinal cells from human pluripotent stem cells remains a major challenge for the development of successful and cost-effective cellular therapies for various forms of blindness. Current differentiation strategies rely upon exposing pluripotent stem cells to soluble growth factors which play key roles during early development (such as DKK-1, Noggin and IGF-1) at 20% oxygen (O_2). This O_2 tension is however, considerably higher than O_2 levels during organogenesis and may impair the differentiation process. In this study, we examined the effect of mimicking the physiological O_2 tension (2%) on the generation of retinal progenitor cells (RPCs) from human induced pluripotent stem cells (iPSCs) and human embryonic stem cells (hESCs). Both cell types were induced to differentiate into RPCs at 20% and 2% O_2. After three days in suspension culture as embryoid bodies (EBs), 2% O_2 caused the activation of hypoxia inducible factor (HIF) responsive genes VEGF and LDHA and was accompanied by elevated expression levels of the early eye field genes Six3 and Lhx2. 21 days after plating EBs in an adherent culture, we observed more RPCs co-expressing Pax6 and Chx10 at 2% O_2. qPCR analysis confirmed that lowering O_2 tension had caused a rise in the expression of both genes compared to 20% O_2. Our results indicate that mimicking

physiological O_2 is a favorable condition for the efficient generation of RPCs from both hiPSCs and hESCs.

1. Introduction

1.1 Photoreceptor dystrophies and possible treatment

1.1.1 Photoreceptor dystrophies

Photoreceptors are light-sensing retinal neurons found in the outer nuclear layer (ONL) of the retina. Two types of photoreceptors, cones and rods, are responsible for vision in the bright and dim lights respectively (Deflyer et al., 2004, Pacione et al., 2003). These specialized neurons carry out phototransduction which converts light into electrical signals in the first step of the visual cycle (Bowmaker and Dartnall, 1980). Dystrophies in photoreceptors result visual impairments and possible blindness in the most severe cases.

Photoreceptor dystrophies are hereditary conditions and characterized by the degeneration of photoreceptors due to genetic defects (Table 1.1). Amongst different types of diseases, retinitis pigmentosa (RP) is the leading cause of photoreceptor degeneration, affecting 1 in every 4000 worldwide (Bundey and Crew 1984). RP is characterized by a progressive degeneration of rod and cone photoreceptors over a period of life time, leaving severely attenuated ONL of the retina where rod and cone photoreceptors normally reside (Hartong et al., 2006). Typical patients with RP experience night vision loss early in their life time (around adolescence) that is followed by the loss of side and central vision as the disease advances (around age 60,

Hartong et al, 2006).

To date, mutations in more than 45 genes have been identified that cause degeneration of photoreceptors. The mutated genes are exclusively found in rod photoreceptors and are the cause their death (Haung et al., 1995). For instance, 4-5% of RP are caused by mutations in α and β subunits of cGMP phosphodiesterase 6 (PDE6A and PDE6B), genes that regulate rod cGMP levels (McLaughlin et al., 1985). When rods are not stimulated by light, a high concentration of cGMP opens cation channels until the release of the neurotransmitter glutamate (Cobbs and Pugh, 1985). Mutations in PDE6A and PDE6B however, cause an abnormal increase in cGMP concentrations in rod outer segment. This prolongs the opening of cGMP-gated cation channels and an excessive flow of cations into the cells from the plasma membrane results in the death of rod photoreceptors by mostly unknown routes (Yamashita and Field 1972).

It has also been reported that the presence of mutated rods themselves is detrimental to surrounding healthy rod photoreceptors. This can be attributed to metabolism and structural interferences and the formation of intracellular protein aggregates (Illing et al., 2002). It is still unknown the cause of cone photoreceptor death when mutations in genes are exclusively found in rod photoreceptors. This highlights the underlying cone photoreceptors' reliance on rod for their survival

which requires further investigation on the interaction between rod and cone photoreceptors for a better understanding of the disease (Leveillard et al., 2004).

Table 1.1

	Retinitis pigmentosa (cone-rod and rod-cone dystrophy)	Progressive cone dystrophy	Stationary cone dystrophy (achromaptosia)	Rod monochromatism	Leber's congenital amaurosis
Description	Poor night vision, deteriorated peripheral (RCD) and central (CRD) vision	Blurred vision, poor colour vision, photophobia	Reduced visual acuity, pendular nystagmus and photophobia	Reduced central vision, poor colour vision and photophobia	Nystagmus, near-absent papillary responses, photophobia and keratoconus
Genes	More than 45 genes have been identified including CRX and ABCR	Several genes including COD1, COD2, GUCA1A and RPGR	L and M opsin genes	CNG3, CNGB3 and GNAT2	CRX, CRB1, GUCY2D, AIPL1, RDH12, RPGRIP1, RPE65, and CEP290
Inheritance	Autosomal dominant Autosomal recessive X linked	Autosomal dominant Autosomal recessive X linked recessive	X linked	Autosomal recessive	Autosomal recessive
Frequency	1:4,000 in the world	1:30,000 in the US	1:100,000 (Men only)	1:50,000 in the world	1:50,000 in the world

Table 1.1 The list of hereditary photoreceptor dystrophies. RP in particular, is a common cause of blindness, affecting 1 in 4,000 people in the world. There is currently no effective treatment available for the above disorders.

1.1.2 Potential treatments for retinal dystrophies

1.1.2.1 Gene therapy

Gene therapy is a therapeutic strategy whereby a normal gene is introduced into the cells to compensate a defective gene that causes diseases. Clinical trials for gene therapy began 20 years ago and encouraging results have been emerging for various genetic diseases in the last 3-10 years (Bainbridge et al., 2008; Cartier et al., 2009; Ponder, 2011). In 2009, a study published in The New England Journal of Medicine reported the outcome of a phase I human clinical trial of gene therapy for one type of hereditary photoreceptor dystrophies Leber's congenital amaurosis (LCA) (Cideciyan et al., 2009). Patients suffering from the disorder have a mutation in RPE65 gene which affects photoreceptors viability and functionality (Hauswirth et al., 2008). In this trial, normal RPE65 gene was delivered into the participants' retina where remaining photoreceptors resided via a recombinant adeno-associated viral (rAAV2) vector. The sensitivity to the dim light increased a few weeks after the treatment and significant visual improvements were sustained up to a year post therapy without any signs of side effects. This human hereditary retinal disorder was the first of its kind to show visual improvement after treatment. The participants are required to be monitored for a full three years for a phase I clinical trials and for another 15 years for health impacts as is required by the FDA for any gene therapy

trials.

It has been reported that neutrophic factors are a possible candidate for therapeutic agent for treating RP (Faktorovich et al., 1990). In particular, ciliary neutrophic factor (CNTF) has been reported to prevent photoreceptor degeneration in animal models of RP (Cayouette and Gravel, 1997; LaVail et al., 1998). Neurotech utilized an encapsulation technology where retinal pigmented epithelium (RPE) cells that were transfected with CNTF were encapsulated with a semipermeable membrane. This allows diffusion of CNTF proteins to the host retina whilst giving protection from the host immune system. Phase I clinical trial reported that this delivery system was reported to be safe and effective in improving vision after transplanting into human eye (Sieving et al., 2006).

1.1.2.2 Cell therapy

The term cell replacement therapy refers to a therapeutic concept that transplants functional cells into organs to replenish damaged or deteriorated counterparts to restore or rescue their functionality. Cell therapy is an attractive candidate for developing treatments for photoreceptor dystrophies as intraocular spaces are known to be immunoprivileged sites (Streilein et al., 2002). In order to develop a successful therapy for photoreceptor dystrophies, transplanted retinal cells

have to survive in the host retina environment and integrate in the ONL of the host retina to form functional synaptic connections for transmission (Yang et al., 2010). Below is a summary of human clinical trial of retinal transplantation for patients suffering from RP.

Table 1.2

Authors	Kaplan et al., 1997	Das et al., 1999	Radtke et al., 1999	Humayun et al., 2000	Radtke et al., 2008
Number of Patients	2	14	2	7	10
Donor cells	Adult photoreceptors	Fetal neural retinal cells	Fetal retinal cells	Fetal retinal cells	Fetal retinal cells and RPE
Transplantation site	Subretinal space	Subretinal space	Subretinal space	Subretinal space	Subretinal space
Safety	safe	safe	safe	safe	Safe
Functional Improvement	None	Not significant improvement in ERG recording	Short term improvements in ERG recording	Short term improvements in ERG recording	On-going

Table 1.2. A summery of up to date human clinical trials for photoreceptor transplantation for patients suffering from RP. ERG (electroretinography) measures electrical responses of various retinal cells (including photoreceptors) in response to light stimuliation. It is a commonly used diagnosis for various retinal diseases.

1.1.2.3 Challenges associated with retinal cell therapy for photoreceptor dystrophies

Almost two decades of human retinal transplantation has not yet achieved satisfactory long term visual improvement in patients with RP. This can be accountable for the lack of the donor cells' ability to integrate into the damaged retina to form functional photoreceptors. Early retinal transplantation studies in animals revealed that integration of donor retinal cells with the host retina for functional recovery has proven much more challenging than ensuring their survival after transplantation. It is often revealed that transplanted retinal cells tend to form rosettes of photoreceptors rather than well-organized layers with the host retina (Gouras and Algvere, 1996; Juliusson et al., 1993). It was later revealed that any functional improvements observed with the transplantation was due to the paracrine effect of survived retinal cells after transplantation (Gamm et al., 2007). Transplanting sheets of photoreceptors has shown to minimize the formation of rosettes in the host retina but this is technically more demanding (Seiler and Aramant, 1998; Silverman et al., 1992).

A breakthrough was made in 2006 when Maclaren et al identified the exact type of transplantable retinal cells that survives and integrate into the host retina of blind mice (MacLaren et al., 2006). By examining cells at different developmental stages (ranged from embryonic day (E)11.5 to adult mice), it was observed that only

post-mitotic immature rod precursor cells (postnatal 1 to 7 days) expressing neural retina leucine zipper (Nrl) transcription factor survived and integrated well into adult or degenerating retina to develop into mature and functional photoreceptors. Transplantation of Nrl-positive cells into the rhodopsin knockout mouse demonstrated improved sensitivity to dim light measured by ganglion cell layer activity compared to the eye that received the sham injection. In addition, the pupil of the eyes that received Nrl-positive cells reacted better to dim light compared to the sham injection group.

In an independent study, researchers have demonstrated that cone precursor cells expressing Crx can also integrate into host retina and form mature rods and cones in animal models of LCA (Lakowski et al., 2010). Crx^+ cells were isolated using FACs from CrxGFP transgenic mice from various developmental stages (E12.5, E13.5, E14.5, E15.5, E16.5, E17.5, P1 and P3). It was revealed that upon transplantation, Crx^+ cells migrated to and integrated in the ONL of the retina to and further developed into mature photoreceptors (except for Crx^+ from E12.5). It was also observed that the integration efficiency was dependent on the stage at which Crx^+ cells were isolated as post-natal cells showed the greatest integrated cells (up to 15,000 cells compared to a few thousands by embryonic Crx^+ cells).

The findings provide a platform to develop an efficient and effective retinal

cell therapy. Failure to sustain long term visual improvements after retinal cell transplantation could be attributed to the heterogeneity nature of the donor cells. Identifying transplantable donor cells as shown by Maclaren et al and Lakowski et al, however would allow isolation and purification of these cells which could enhance efficacy of transplantation.

It would be exciting to observe whether transplantation of Nrl^+ and Crx^+ cells in patients with genetic photoreceptor dystrophies could sustain long term visual improvements (Figure 1.1). To generate Nrl^+ and Crx^+ cells that are suitable for human clinical application, a novel strategy has to be developed that avoids creating transgenic organisms for obvious ethical reasons. It is widely believed that Nrl^+ and Crx^+ cells could be generated from pluripotent stem cells, unspecialized cells with a potential to become almost any types of cells in the body. (Fig 1.1).

Figure 1.1

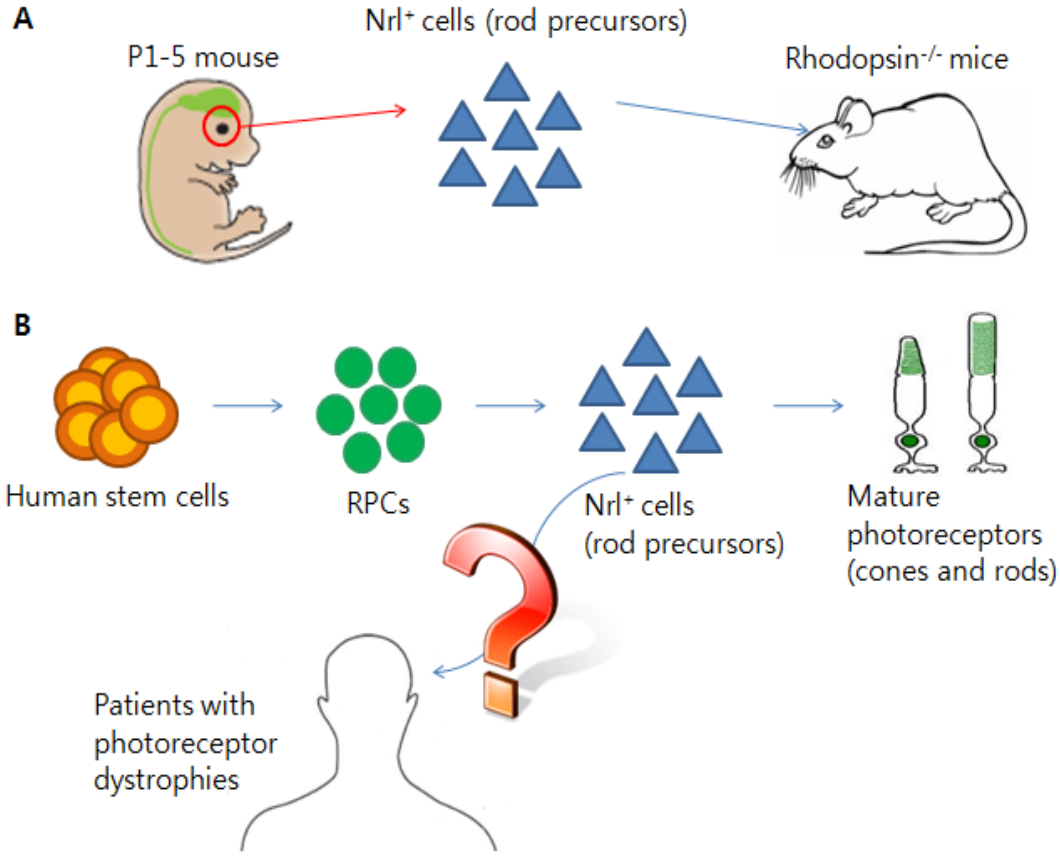


Figure 1.1. Current and future retinal transplantation strategies. (A) Maclaren et al., demonstrated the isolation of Nrl^{+} rod precursors from postnatal mouse that formed new photoreceptors and improve vision after transplantation into the blind mouse model. (B) Possible human retinal transplantation strategy using cells derived from human stem cells instead of aborted fetus.

1.2. Types of human Stem Cells

1.2.1. Human Embryonic stem cells (hESCs)

1.2.1.1. hESCs culture

hESCs are a unique population of cells in that they self-renew themselves without adopting specific functions. Due to their uncommitted nature, they are thought to be pluripotent, capable of becoming almost all types of cells in the body (Evans and Kaufman, 1981). The first hESCs line was established in 1998 by the Thomson group from Wisconsin University (Thomson et al., 1998). hESCs are created from the surplus *in vitro* fertilized embryos originally created as part of *in vitro* fertilization (IVF) treatment. These embryos are donated for research purposes by parental donors under informed consent.

The inner cell mass (ICM) are isolated from the blastocyst and cultured in the presence of mouse embryo fibroblasts (MEFs) with supplement of bFGF to maintain pluripotency (Thomson et al., 1998). MEFs are regarded as "feeder cells" as they provide nutrients and unknown growth factors that are thought to support pluripotency in the culture. This is the classic maintenance culture condition although hESCs can also be maintained in the presence of human embryo fibroblasts (hEFs) or in the "feeder-free" conditions using commercially available matrices (Hernandez et al., 2010). These culture methods eliminate the possibility of cross-contamination between the two species which are crucial for hESCS clinical applications. During in

vitro culture of hESCs, it is a frequent laboratory practice to examine whether hESCs exhibit all the fundamental properties of true hESCs. This process is often known as characterization and commonly used methods to characterize typical properties of hESCs are summarized below in Table 1.3.

Table 1.3

Properties	Methods	Results
Undifferentiated state	Immunocytochemistry	Surface marker expression SSEA3 and 4, Tra-1-60 and 80
Pluripotency	Immunocytochemistry	Transcription factors Oct4, Nanog, Sox2, Rex1 and UTF1
	EB formation (followed by immunocytochemistry)	Three embryonic germ layers marker expressions
	Teratoma formation in SCID mice (followed by immunohistochemsitry)	Tissues expressing germ layer markers
	Chimera formation*	Formation of chimera offspring
Normal chromosomes	Karyology	Correct numerical and structure of chromosomes
Proliferative capacity	Prolonged in vitro culture	Ability for a long term in vitro culture whilst maintaining pluripotency and correct number and structure of chromosomes

Table 1.3 Characterization of hESCs. hESCs are often characterized during in vitro culture using a various laboratory techniques. * Due to ethical reasons, chimera experiments with mammalian ESCs are not permitted.

1.2.1.2 hESCs differentiation

Since the establishment of the first hESCs lines in 1998, intense research interests have been focused on generating specific cell types that are functional and capable of restoring damaged counterparts *in vivo* after transplantation. Pluripotent hESCs can be coaxed to become specific types of cells. The process in which uncommitted cells adopt specific characteristics or functionality is termed "differentiation". Enrichment of certain types of cells by differentiation is critical as transplantation of pluripotent stem cells cause tertatoma *in vivo* (Fukuda et al., 2006; Choo et al, 2009). Traditionally, differentiation of hESCc involves the formation of embryoid bodies (EBs) in suspension that triggers differentiation of pluriopotent stem cells into three germ layers (Thomson et al., 1998). These aggregates of cells are then plated in the presence of growth factors and small molecules to direct differentiation into a specific lineage of cells. The addition of growth factors in the differentiating cultures mimics the signaling pathways during the development of the corresponding types of cells.

For instance, it was reported that the generation of pancreatic β-cells (endoderm lineage cells) is possible from spontaneously differentiating EBs but only 1-3% of the cells were insulin-expressing cells (Assady et al., 2001). This is however significantly increased when EBs were cultured adherently in the presence of insulin-transferrin-selenium-fibronectin medium, followed by supplementation with N2, B27,

and bFGF. 11 days post plating EBs in the growth factors revealed up to 70% insulin-expressing cells in the culture (Segev et al., 2004).

Many mesodermal lineage cells are possible candidates for various degenerative diseases. For instance, blood forming hematopoietic cells can be generated from hESCs which can then subsequently be used to treat patients with leukemia (Ledran et al., 2008). This differentiation has been achieved by supplementing interleukin 3 and interleukin 6, in combination with BMP4 after EB formation (Chadwick et al., 2003). In addition, it has been reported that cardiomyocytes, which could be used to treat cardiac diseases such as myocardial infarction, can be generated from hESCs using activin-A and bone morphogenic protein 4 (BMP4, Laflamme et al., 2007). Bone and cartilage cells, potential therapeutic agents for osteoporosis and osteoarthritis, are differentiated using small molecules such as ascorbic acid, β-glycerophosphate and dexamethasone (Sottile et al., 2003).

Generating functional neurons for cell therapy is a subject of intense investigation. The first evidence of neural differentiation was demonstrated in 2001 when neural progenitor cells (NPC) were generated and expanded in the presence of B27, epidermal growth factor (EGF) and basic fibroblast growth factors (bFGF, Reubinoff et al., 2001). Retinoic acid (RA) is a commonly used small molecule for

induction of various neurons. hESCs-NPCs were also capable of differentiating into dopaminergic neurons in the presence of RA, FGF8 and sonic hedgehog (SHH) (Perrier et al., 2004) and oligodendrocyte in the presence of bFGF and EGF which are currently in Phase I human clinical trial for the safety of the cells for spinal cord injuries (Nistor et al., 2005). Specific populations of cells from hESCs can also be achieved through co-culture systems with tissues derived from other species or through genetic manipulation. These methods are efficient in generating specific cells from hESCs but they have been regarded as not suitable for therapeutic purposes due to safety issues concerning with viral vectors and cross contamination (Li et al., 2008).

1.2.1.3 Issues associated with hESCs

In 2009, a ground-breaking milestone was achieved when Geron announced the first ever human clinical trial approval for hESCs-based therapy for treating spinal cord injuries (Table 1.4). This was followed by Advanced Cell Technology (ACT) who began their human clinical trial for the treatment of age-related macular degeneration (AMD) using retinal pigment epithelium derived from hESCs (Table 1.3). Despite promising advances, hESCs-based cell therapy faces major challenges that are still to be overcome to become successful and viable treatment.

It is well documented that allerogenic transplantation of hESCs-derived cells raises concerns with immunogenicity (Drukker and Benvenisty, 2004). This can be avoided, to some extent, by administrating systemic immunosuppressive drugs but this could leave patients to venerable to opportunistic infections (Choumerianou et al., 2008). It has been reported that engraftment of hESCs-derived cells is not required to exert therapeutic effects. In this study, hESCs-derived endothelial cells (hESCs-ECs) were encapsulated with Matrigel and implanted into a mouse model of hindlimb ischemia. The implantation of encapsulated hESCs-ECs was not engrafted in the host tissue but showed functional improvements (measured by blood flow in the ischemic region) through a paracrine effect of secreted angiogenesis-related factors (Moon et al., 2011(b)). Other strategies to overcome immunogenicity of hESCs include creating patient-specific hESCs through somatic nuclear transfer

(Wilmut et al.,1997) and creating induced pluripotent stem cells (iPSCs, which are explained in depth in the following chapter).

Regardless of hESCs differentiation protocols, all hESCs differentiation cultures are always heterogeneous. Among the presence of different types of cells, residual pluripotent hESCs or proliferating progenitor cells cause tumour formation after transplantation. For this reason, it is a prerequisite to remove tumorigenic cells in the cultures prior to transplantation for safe treatment. The most common technique to create cultures free of tumorigenic cells is using fluorescence activated cell sorting (FACS). This can be achieved through either isolating tumorigenic cells (negative sorting) or specific cell populations (positive sorting) based on the expression of cell surface markers. For instance, isolating Sox1-positive cells and subsequent transplantation into the striatum did not form teratoma 8 weeks post transplantation (Fukuda et al., 2006). For cell types that do not have known cell surface markers specific for them, the negative sorting is a preferred method (Choo et al., 2008). Other methods include the use of pharmacological agents that target the dividing cells. For instance, N-oleoyl serinol specifically induces apoptosis of pluripotent stem cells in the differentiation culture (Bieberich et al., 2004).

Along with safety issues associated with hESCs-based therapy, ability to generate a large number of functional cell types from hESCs remains one major

hurdle for efficient and economical therapy. hESC differentiation strategies mentioned in the previous section are designed based on the studies of the developmental event of tissues during the early human development. Mimicking these microenvironment during hESC *in vitro* differentiation however, has not been proven to be effective as many aspects of developmental studies remain unknown due to a sheer complexity of organogenesis. This often results in lengthy hESCs differentiation protocols generating a low number of desired cell types. For instance, it has been reported that hESCs can be directed to differentiate into cells with pancreatic phenotypes but only a small percentage of those cells meet the criteria for functional pancreatic beta cells (Naujok et al., 2011). It has been reported that a large number of functional neuronal cell types could be produced using a combination of feeder cells, growth factors and genetic manipulation (Li et al.,2008). However, the use of animal derived cells and gene modification are not suitable for human clinical applications (Choumerianou et al., 2008). Therefore developing efficient yet clinically acceptable differentiation protocol remains a monumental challenge for a therapy that is efficient and cost-effective.

Other issues related to hESCs-based therapy include the lack of engraftment of hESCs-derived cells after transplantation for functional recovery, ethical issues relating the destruction of embryo for derivation of hESCs lines and possible cross

contamination from animal derived medium components (Correia et al., 2005, Lo and Parham, 2009).

Table 1.4

Company	Medical condition	hESCs line	Transplantable cell identity	Clinical Trial	Transplantation/Effect
Geron	Spinal cord injury	H1	Oligodendrocyte progenitor cells (OPCs)	Phase I (2010)	OPCs are directly injected into the site of injury Remylination of damaged axons in the spinal cord
Advanced Cell Technology (ACT)	Dry Age-related macular degeneration (Dry AMD) Stargardt's Macular Dystrophy	MA09	Retinal Pigmented Epithelial (RPE) cells	Phase II (2011)	Subretinal transplantation Prevents the loss of RPE population in the retina
UCL/Pfizer	All types of age-related macular degeneration (AMD)	N/A	Retinal pigmented Epithelial (RPE) cells	Phase I (2012)	Subrenital transplantation Prevention of RPE loss

Table 1.4. Clinical application of hESCs-based cell therapy for degenerative conditions.

1.2.2. Human induced pluripotent stem cells (iPSCs)

1.2.2.1 iPSCs technologies

iPSCs are ESCs-like pluripotent stem cells that are derived by transfection of a combination of genes in somatic cells (Takahashi et al., 2007; Yu et al., 2007). iPSCs are cultured in the same way as hESCs and exhibit many characteristics of hESCs such as the expression of stem cell markers, proliferative capacity, teratoma formation and the ability to differentiate into the three embryonic germ layers (see section 1.1). Despite reported similarities between the two cell types, a full extent of analysis is still required to determine whether it can be a direct substitute for ESCs.

The first human iPSCs lines were created by two independent groups in 2007 (Takahashi et al., 2007; Yu et al., 2007). Both groups demonstrated reprogramming of human somatic cells by delivering a combination of four transcription factors (Yamanaka factors: Oct4, Sox2, Klf4 and c-Myc and Thomson factors: Oct4, Sox2, Nanog and Lin28) required for pluripotency using retroviral vectors. This method however has become less popular due to concerns over potentially activating cancer-causing genes (oncogenes) introduced in the host genome by the viral vectors. In order to avoid the complications of potential cancer formation, other researchers have used alternate gene delivery systems. Stadtfeld et al used an adenovirus to introduce a combination of genes to reprogramme mouse skin cells into iPSCs (Stadtfeld et al., 2008). The significant advantage of using

adenovirus is that it does not integrate any of their own genes into the host genome, minimizing the risk of causing mutation of the host genome. Despite ensuring safety of iPSCs, their creation using this technique compromises efficiency as its yield is 100-fold lower than the one describe in Yamanaka and Yu studies.

Following from their initial study of reprogramming using retroviral vector, Yamanaka et al demonstrated another ground-breaking iPSCs technology by which mouse skin cells were reprogrammed into iPSCs without using a vector (Okita et al., 2008). In this study, cells were transfected with two plasmids, one carrying c-Myc and the other carrying the rest of the factors Oct4, Sox2 and Klf4. Although this method avoids using viral vectors, this technology still uses cancer-causing gene c-Myc and suffers from a low efficiency.

The technique which generates clinically more friendly iPSCs at reasonable efficiency has been described using transposon-based vectors. Using such vectors, integrated genes can be removed after completion of reprogramming, leaving the host genome unaltered and free of oncogenes (Kaji et al., 2009). Using piggyBac transposons, researchers constructed a vector with a single expression cassette containing the four reprogramming factors. The vector was then introduced into mouse embryonic fibroblasts with transponase which yielded iPSCs with a comparable efficiency to integrating vectors (Yusa et al., 2009). By sequencing the

integration site of piggyBac vector, it was revealed that the vector was successfully excised from the host genome. In addition, they further accessed pluripotency of resulting iPSCs by teratoma and chimera experiment.

In 2009, Ding et al developed a novel iPSCs technology that relies on the delivery of proteins to the somatic cells instead of transcription factors (Zhou et al., 2009). In this study, authors postulate that delivering reprogramming proteins directly to somatic cells avoids potential tumourgenesis. In order for proteins to pass through the cell membrane, the four reprogramming factors were tagged with a poly-arginine protein transduction domain and expressed in inclusion body of E.coli which were solublized, refolded and purified. This iPSCs technology has major advantages over previous ones that involve delivering transcription factors. The most significant advantage is that protein delivery eliminates the risk of developing cancer as it does not modify the host genome.

Figure 1.2

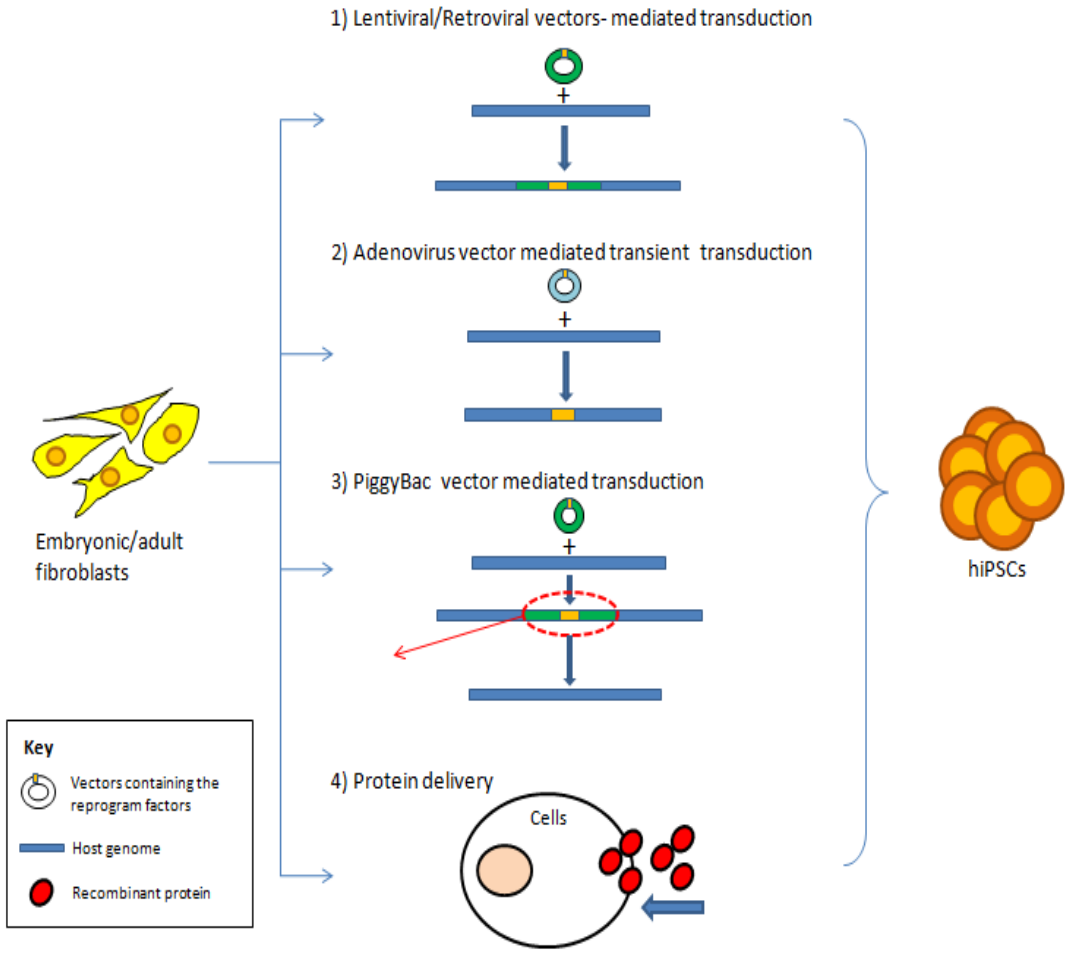


Figure 1.2. Current iPSCs technologies.

1.2.2.2 Application of iPSCs

Production of iPSCs has raised the hope for personalized cell replacement therapy. In theory, such a practice would involve isolating skin cells from a patient, reprogram into iPSCs, then differentiate into a certain cell type before putting back into the patients. In addition, such transplantation is autologous, circumventing the need to accompany immunorepressive drugs with the treatment (Amabile and Meissner, 2009). This concept has been demonstrated when researchers created iPSCs from a patient suffering from a neurodegenerative condition named amyotrophic lateral sclerosis (ALS) which were then differentiated into motor neurons that are destroyed by the disease (Mine-Neto et al., 2011). This potential clinical application of iPSCs is considered as a long term application which requires more fundamental investigation and validation. More immediately, iPSCs could provide an important tool for producing a test bed for toxicology test for drug development and modeling human diseases to understand underlying mechanisms (Heng et al., 2009; Yokoo et al., 2009).

Figure 1.3

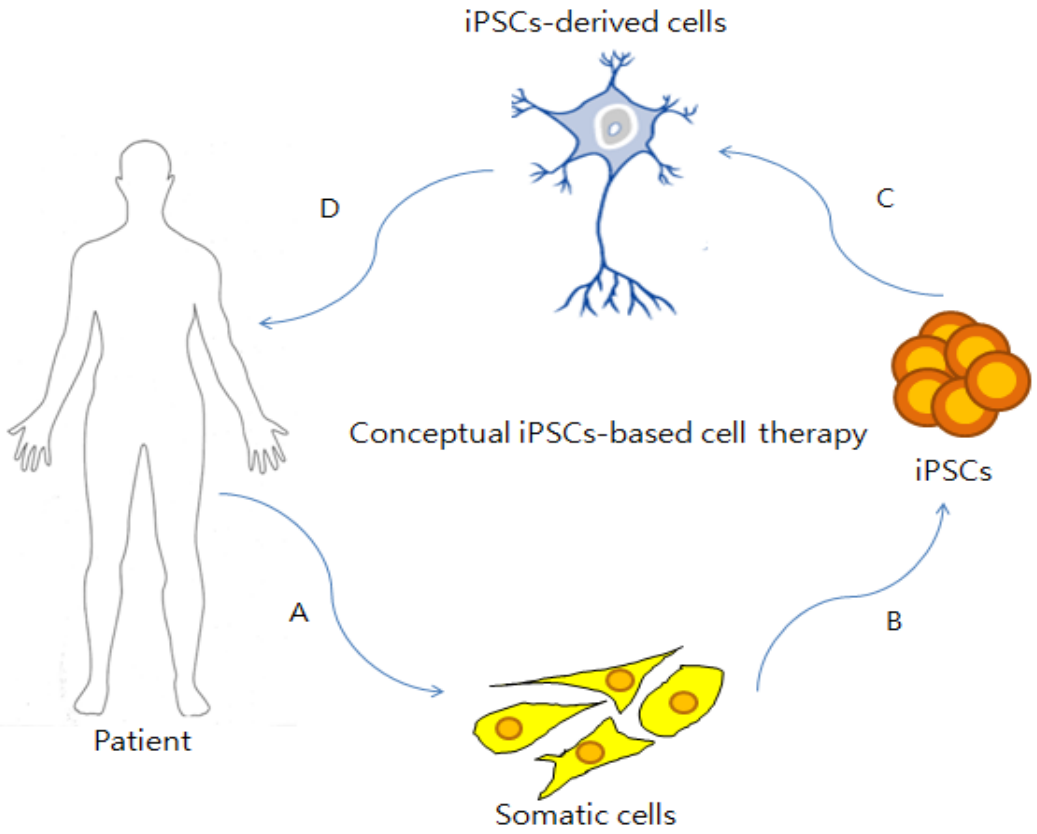


Figure 1.3. Conceptual iPSCs-based cell therapy. (A) Somatic cells are isolated from the patient. (B) Somatic cells are reprogrammed into iPSCs using above-mentioned technology. (B) Subsequent iPSCs are induced to differentiate into functional specialized cells. (B) iPSCs-derived cells are transplanted back into the patient.

1.2.2.3 Challenges for iPSCs

As far as therapeutic application of iPSCs is concerned, the first hurdle that needs to be overcome is developing technologies that robustly produce safe iPSCs. Reprogramming cells using viral vectors is potentially tumorigenic due to the presence of the integrated oncogenes in the host genome. Technologies for creating safer iPSCs have been developed using piggyBac transposons or protein delivery systems but their efficiency still remains to be optimized as currently yield is around the region of 0.1-1% (Yamanaka et al., 2007, Zhou et al., 2009).

iPSCs therapy would have advantages over hESCs in that they are ethical and patient-specific which eliminates the risk of immune rejection. Despite iPSCs sharing many similar characteristics with hESCs, these two cells lines are fundamentally two different types of cells. Studies have shown differential expression level of many genes between the two cell types and noticeable differences in epigenetic methylation also have been observed (Lister et al., 2011). However, it has also been reported that variation in the gene expression profiles between hESCs and iPSCs is no more than the variability observed between individual hESC lines (Guenther et al., 2010). Whether these differences are consequential in terms of functionality and safety concerns, are subject to further investigation (Li et al., 2011). Recently, one study has reported that iPSCs can provoke immune rejection upon transplantation, suggesting that more transplantation studies are also required for the

safety of possible iPSCs-based therapy (Zhao et al., 2011).

Despite circumventing problems associated with immunogenicity, transplantation of iPSCs-derived cells is still susceptible to tumour formation. It is believed that directly reprogramming somatic cells to functional specialized cells without the intermediate pluripotent stage reduces the risk of forming teratoma. This concept was first described by Vierbuclen et al. In this study, researchers screened numerous fate inducing factors and revealed that a combination of Brn2, Ascl1 and Myf1l (BAM combination) efficiently generates functional neurons from mouse embryonic and postnatal fibroblasts (1.8-7.7%, Vierbuclen et al., 2010). This lentiviral vector-mediated transduction generates induced neuronal (iN) cells expressing typical pan neuronal markers MAP2, TuJ1 and NeuN. In addition, these cells were shown to generate action potentials measured by electrophysiological recording. The BAM combination was also capable of generating iN cells from human fetal fibroblasts as early as 7 days after transduction (Pang et al., 2011). Despite exhibiting neuronal morphologies, these cells were functionally immature. This prompted screening of further 20 factors in search for a factor that could enhance the generation of iN cells in combination with the BAM. It was revealed that NeurD1 with BAM combination generated mature iN cells and that expressed pan neuronal markers Tuj1, NeuN and NCAM. In addition, these human iN cells were

shown to generate action potentials around 35 days post transduction. In addition to directly reprogramming functional neuronal cells from fibroblasts, it has been also described that functional cardiomyocytes can be directly reprogrammed from postnatal cardiac or dermal fibroblasts (Ieda et al., 2010). Researchers in this study used a combination of Gata4, Mef2c and Tbx5 to generated 30% induced cardiomyocytes (iCMs) expressing cTnT markers that were also exhibiting spontaneous contraction.

1.2.3 Adult Stem Cells (ASCs)

Adult stem cells (ASCs) reside in tissues in the body and are responsible for repairing and replacing damaged cells or tissues of organs throughout the life time (Barrilleaux et al., 2006). The list of organs that contain stem cells population continues to grow that includes bone marrow, brain and skeletal muscle (Table 1.5). ASCs are characterized by their ability to self-renew for the life time of the organism. This cannot be analyzed *in vivo* for complex organisms like human but *in vitro* assays have been shown their proliferative capabilities (Fernando et al., 2010).

Another property that characterizes ASCs is their ability to differentiate into multiple types of specialized cells (Davis and Temple, 1994; Bianco et al., 1999). Traditionally, it has been believed that ASCs are multipotent stem cells, giving rise to limited types of specialized cell of the tissue in which they reside. For instance, hematopoietic stem cells (HSCs) from bone marrow differentiate into all types of blood cells to replenish aged blood cells and neural stem cells (NSCs) found in adult brain tissues have been shown to produce three types of nerve cells (neuron, astrocyte and oligodendrocyte) depending on the signal they received (Gage et al., 1995). This limited differentiation capacity of ASCs restricts their therapeutic application to only certain types of degenerative medical conditions (Kocher et al., 2001; Shiabuddin et al., 1999). It is not until recently when studies have shown that ASCs can generate specialized cell types of other tissues that either generated from

the same embryonic germ layer or from a different germ layer (Prockop, 2003). For instance, studies have shown that NSCs isolated from adult brain tissue could generate hematopoietic cells and reciprocally, bone marrow derived stem cells were shown to differentiate into neural tissues (Mezey et al., 2000; Woodbury et al., 2000). Their ability to differentiate into specialized cell types of other embryonic germ layer often referred as "transdifferentiation" which excites the scientific community for their potential wider applications in cell replacement therapy.

ASCs transplantation is autologous, harvesting ASCs from tissues from individuals which are then induced to differentiate into a specific cell type *in vitro* before placing back into them. This type of transplantation has a critical advantage over ESCs-based transplantation as this lessens the complication of immune rejection and the possibility of developing tumour-like formation, as well as avoiding ethical issues associated with ESCs-based therapy (Cultler and Antin, 2001). Despite promising evidence of transdifferentiation capacity of ASCs, there are still lingering doubts over whether ASCs are capable of generating functional mature cell types of other embryonic germ layers. One study demonstrated that born marrow-derived stem cells (BMCs) are capable of differentiating into retinal neurons (GFAP, calbindin, rhodopsin and vimentin) upon transplantation into the intravitreous space, but failed to either integrate with the host retina or further develop into mature

photoreceptors (Tomita et al., 2001).

Table 1.5

Source	Cell type	Function
Bone marrow (Wilson and Trumpp, 2006)	Hematopoietic stem cells (HSCs)	Differentiate into all types of blood cells
Skeletal muscle (Chen and Goldhamer, 2003)	Skeletal muscle stem cells	Repairs degenerating muscle fibres
Brain (Vescovi et al., 1999)	Neural stem cells (NSCs)	Differentiate into neurons, astrocyte and oligodendrocyte
Dental pulp (Shi et al., 2005)	Mesenchymal stem cells (MSCs)	Differentiates into osteocytes, chondrocytes and adipocytes
Cornea (Dua and Azuaza-Blaco, 2000)	Limbal stem cells (LSCs)	Regeneration of limbal epithelial of the cornea
Bone marrow (Pittenger et la., 1999)	Mesenchymal stem cells (MSCs)	Differentiate into adipocytes, cartilage, bone
Pancreas (Bonner-weier and Sharma, 2002)	Pancreas stem cells	Differentiate into insulin-producing beta cells

Table 1.5. Sources for adult stem cells in the body and their functions.

1.3. Retinal differentiation of human pluripotent stem cell

1.3.1. Differentiation strategies

The retinal differentiation of iPSCs and hESCs is largely based on mimicking events taking place during mammalian retinal development by supplementing key pathway agonists or antagonists. During early mammalian development, antagonism of bone morphogenetic protein (BMP) and Wnt signaling pathways reportedly plays significant roles in defining telencephalon of the forebrain (Mukhopadhyay et al., 2001). Following the forebrain specification, the formation of optical vesicles (OV) is induced from the diencephalon of the forebrain as it bilaterally evaginates and expands through mesenchyme towards the surface ectoderm (Adler and Canto-Soler, 2007). The contact between the OV and the ectoderm triggers complex structural changes as the surface ectoderm thickens and becomes a structure called a lens placode, which eventually invaginates the OV and forms a two-layered optic cup (OC) and lens vesicle (Fuhrmann et al., 2000). The key cellular fate decision of neural retina versus the retinal pigment epithelium (RPE) in the developing OC is regulated by the inductive signals emanating from the surrounding extraocular tissues (Saha et al., 1992; Chow and Lang, 2001).

The presumptive retina is initially found on the thick inner wall of the cup, in close contact with the surface ectoderm (Dragomirov 1937). Growth factors such as fibroblast growth factor (FGF) and sonic hedgehog (Shh) from the surface ectoderm

have been reported to induce the differentiation of the OC into retina lineage cells, which express neural retina-specific transcription factors Chx10 and Pax6 (Pittack et al., 1997). Extraocular mesenchyme, on the other hand, promotes the differentiation of the presumptive RPE on the thin outer layer of the OC, possibly under the influence of activin-like signals (Fuhrmann et al., 2000; Kagiyama et al., 2005). The pigmented cells express RPE specific marker Mitf and was shown to inhibit the expression of Chx10 and Pax6 (Fuhrmann et al., 2000). The cells found in the OC are generally regarded as retinal progenitor cells (RPCs) and have the ability to differentiate into the retinal neurons in a sequential manner by producing ganglion cells, cone photoreceptors and horizontal cells first, followed by amacrine cells and rod photoreceptors and lastly bipolar and Muller glia (Altshuler et al., 1991).

Figure 1.4

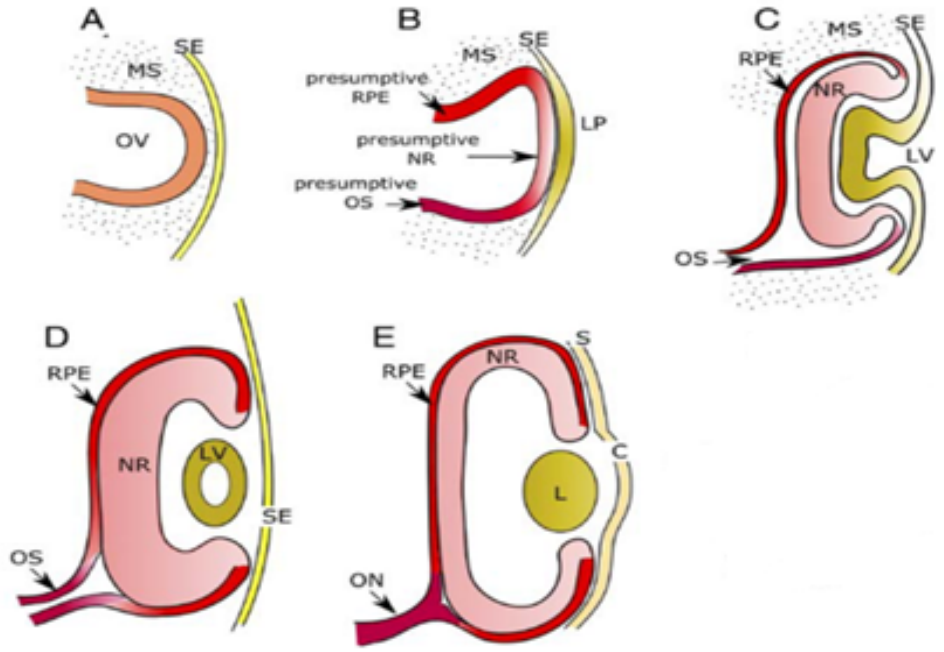


Figure 1.4. Schematics of key development features during mammalian retina development. (A) The formation of optic vesicle from the diencephalon. (B) Specification of the optic vesicle into presumptive RPE, neural retinal (NR) and optic stalk (OS) upon contact with the surface ectoderm,. (C) The formation of optic cup and lens vesicle. (D and E) Transition from early to mature optic cup. Lens vesicle becomes a solid structure as optic cup matures and RPE and NR becomes opposed. Optic stalk becomes optic nerve (Alder and Canto-Soler, 2007).

1.3.2. Tomas Reh Group, Wisconsin University, USA

A group of researchers from Wisconsin University in 2006 first demonstrated the production of hESCs-derived retinal cells and their ability for functional recovery in blind mice (Lamba et al., 2006). Lamba et al developed an efficient protocol which generates almost 80% of RPCs co-expressing typical RPC markers Pax6 and Chx10 from H1 hESCs line. The protocol uses soluble growth factors DKK-1 and Noggin to antagonize Wnt and BMP signal pathways respectively which mimic the forebrain formation during the early development (See section 1.1.1). In addition, they showed that supplementing Insulin-like growth factor 1 (IGF-1) specifically enhances RPC lineage differentiation in favour of other anterior neuronal lineages. H1 hESCs were cultured as EBs for three days in the media supplemented with DKK-1, Noggin and IGF-1 as well as N2 and B27 supplements which are widely used for neural induction. The subsequent EBs were plated on poly-d-lysine/matrigel coated dishes with the same media as well as bFGF which enhances photoreceptor development (Hicks and courtois, 1992). The adherent differentiation of EBs was cultured for 21 days which resulted in ~80% of colonies co-expressing Pax6 and Chx10, quantified by immunoreactivity to the markers. It was also revealed 21 days after differentiating hESCs yielded cells expressing retinal neurons expressing PKCα (bipolar marker), Prox-1 (horizontal cells), Tuj1 (Ganglion cells), Neurofilament-M (Amacrine cells). In addition, it was also shown by

immunocytochemistry that immature (Nrl and Crx) and mature (s-opsin and rhodopsin) photoreceptor markers were present in the culture although their population was very low. In order to examine whether immature photoreceptors could differentiate into mature phenotype, they co-cultured hESCs-derived retinal cells with retinal explants from adult mice and observed integration and differentiation into mature photoreceptors in the explants.

This retinal differentiation protocol was validated with several iPSCs (iPSC-MHF2 c1 and c2) and hESCs lines (Hues6, 14 and 16 and Mel1 and 2) (Lamba et al., 2010). It was revealed that the differentiation protocol was robust as each cell lines behaved with a minor variation in the expression level of Crx, although iPSCs-MHF2 c2 was found to be less effective amongst them all.

This retinal differentiation of iPSCs and hESCs has been the base for other retinal differentiation studies such as that of Amirpour et al (2001) and shown to be effective in inducing retinal differentiation with mouse iPSCs (Tucker et al., 2011).

1.3.3 Takahashi Group, Riken University, Japan

The group developed the first differentiation protocol that induces neural retinal lineage differentiation from mouse ESCs (mESCs) (Ikeda et al., 2005). The authors investigated a wide spectrum of pathway antagonists during EB cultures and observed that the combination of DKK-1, Lefty-A (nodal antagonist), Activin-A and 5% FCS (also known as SFEB/DLFS culture) was effective in inducing retinal differentiation yielding 16% of RPCs co-expressing Pax6 and Rx. The percentage of cells expressing mature photoreceptor markers was very low (~0.5% of the culture) but this was significantly enhanced when co-cultured with embryonic retinal culture (~30% of the culture). In 2008, the group developed the first chemically-defined culture condition that avoids the use of co-culture system with animal derived tissues (Osakada et al., 2008). The SFEB/DLFS culture was optimized to produce RPCs from hESCs. EBs were cultured for 20 days without the presence of FCS but in the presence of Dkk-1 and Lefty-A. EBs were then plated on poly-D-lysine/laminin/fibronectin-coated dishes and by day 35, 16% of cells co-expressed RPCs markers Rx and Pax. To determine whether RPCs could further differentiate into mature photoreceptors, the adherent culture was extended and photoreceptor precursors expressing Crx marker was observed on day 90. The percentage of Crx-positive cells increased to 11.3% on day 120 and 20% on day 130 when RA and taurine, chemical that are known to involve in the photoreceptor development, were

supplemented in the culture on day 90. In addition, on day 200, the culture also contained 8.5% rhodopsin positive cells which co-expressed recoverin. The expression of cone markers such as red/green opsin (8.9%) and blue opsin (9.4%) was also present at the same time point. This protocol was validated with three iPSCs lines (201B6, 201B7 and 253G1) and produced RPCs with a comparable efficiency (~20%) but one iPSCs line (210B6) failed to differentiate into RPCs (Hirami et al., 2009). The other two lines however, further differentiated into Crx-positive cells (14%) on day 90. Upon the treatment with RA and taurine at this time point yielded 13% recoverin and rhodopsin co-expressing cells on day 120. In the following study, the group demonstrated that growth factors, which are normally produced in animals/E-coli, can be substituted with soluble small molecules (Osakada et al., 2009). The use of small molecules has a number of advantages over growth factors in that they minimize cross-species contamination, small lot-to-lot variation and low cost. Chemical inhibitors such CK1-7 and SB-431542 antagonise wnt and nodal pathway respectively and induces retinal differentiation with a comparable efficiency to Dkk-1 and Lefty-A induced differentiation of iPSCs. This differentiation protocol was verified by an independent group who used the same combination of growth factors to derived RPCs from hESCs (Nistor et al., 2010).

The group is the first to develop a chemically defined media composition for generating mature photoreceptors without the use the co-culture system with animal explants. For this reason however, differentiation efficiency has been compromised as the protocol takes up to 180 days to generate mature photoreceptors. Future studies are required to efficientlty generated photoreceptors in the chemically defined media.

1.3.4. Transplantation of hESCs- and iPSCs- derived retinal cells in animal models

In order to demonstrate whether hESCs- or iPSCs-derived retinal cells are clinically suitable for treating photoreceptor dystrophies, Lamba et al first performed a subretinal transplantation of GFP-labelled hESCs-derived retinal cells into a mouse model of degenerating retina (Crx knockout mice) (Lamba et al., 2009). Within 2 to 3 weeks of transplantation, electroretinalgraphic (ERG) analysis revealed that mice that received transplantation demonstrated a clear response to a flash of light. This suggests the formation of functional photoreceptors in the retina upon transplantation and indeed, histological examination of the eyes that received transplantation revealed the expression of rod photoreceptor markers recoverin and rhodopsin. Additionally, the transplanted hESCs-retinal cells expressed synaptic markers synaptophysin and PSD95 (postsynaptic density protein 95). This transplantation strategy relies on the transplantation of a mixture of cells derived from hESCs which could bring adverse side effects such as teratoma formation. In the following study, in order to develop a safe and efficient transplantation, the group transfected hESCs- or iPSCs- derived retinal cells with a lentivirus expressing GFP under the promoter of human inter-photoreceptor binding retinol binding protein (IRBP). IRBP is expressed during the early development of rod and cone photoreceptors and been reported to drive photoreceptor-specific genes in transgenic mice (Pepperberg et al.,

1993). For this reason, retinal culture was transfected with IRBP-GFP in order to purify retinal cells that are capable of forming photoreceptors from contaminating cell populations in the culture. hESCs and iPSCs were transfected with the construct 4 weeks after undergoing retinal differentiation. The retinal cell culture was FACS-sorted based on the GFP expression and over 90% of the sorted population expressed Nrl, Crx and recoverin. Upon transplantation, IRBP-expressing cells integrated with the retina of wild type mice and further differentiated into mature photoreceptors expressing Otx2, rhodopsin and recoverin. It still remains unclear however, whether IRBP-expressing cells can survive and demonstrate functional integration upon transplantation into a mouse model with degenerating retina. In addition, in order for this to be viable for human application, purification of transplantable cells based on the surface markers expressions is prerequisite.

Retinal cells derived from mouse iPSCs have been shown to improve vision upon transplantation into rod deficient model of mouse ($Rho^{-/-}$ mouse). In this study, using FACs sorting, researchers removed residual proliferating cells from the retinal cultures derived from mouse iPSCs. Transplantation of this retinal culture showed no teratoma formation after 3 weeks of transplantation compared to transplantation of mixture culture which resulted in teratoma formation in 2 out 6 mice they transplanted (Tucker et al., 2011). The transplanted cells migrated to the ONL of the

retina where 6.4% of the cells integrated to develop the proper photoreceptor morphologies. In addition, the integrated cells expressed markers specific for rods (recoverin and rhodopsin), cones (blue/green opsin), rod outer segment (ROM-1) and synapse (synaptophysin). To analyse functional improvements after transplantation, ERG analysis of $Rho^{-/-}$ mice that received transplantation revealed an improvement in the b-wave amplitude 21 days after transplantation. In addition, the expression of nuclear c-FOS was observed in the interneurons of the inner nuclear layer (INL) which is produced when photoreceptors are stimulated by light (Huerta et al., 1997).

1.3.5. Futures for hESCs- and iPSCs- derived retinal therapy for patient suffering photoreceptor dystrophies

Above described studies raise the hope of developing a feasible hESCs- or iPSCs-based treatment for photoreceptor dystrophies. In order for this to be clinically acceptable for human trials, a suitable purification method for transplantable retinal cells is required. So far, purification of donor cells derived from iPSCs involves gene modification which is not acceptable for human use (Lamba et al., 2010). Although transplanting hESCs-derived retinal cells have shown to improve vision in animal models of LCA, this approach has the risk of developing teratoma by residual proliferating cells in the culture (Tucker et al., 2011). In order to overcome this problem, efforts have been made to find novel cell surface antigens specific for photoreceptor precursor cells. Sorting cells based on the surface antigen allows purification of a particular cell population without damaging the cells. Koso et al., observed that the expression of CD73 was absent during embryonic stage but increased dramatically until postnatal day 9 (Koso et al., 2009). These CD73 expressing cells did not express the proliferating marker Ki67, demonstrating that $CD73^+$ cells were postmitotic cells. Further analysis with immunocytochemistry revealed that the majority of $CD73^+$ cells co-expressed with rhodopsin but did not with PKCα (bipolar marker). In addition, $CD73^+$ cells exclusively expressed Nrl and Crx markers but not RPC markers such as Chx10 and Hes1. In addition, it was

revealed that transplantation of $CD73^{+}$ enriched cells integrated better in the ONL of the retina after transplantation compared to retinal cells acquired from P0 rhoEGFP mice (Eberle et al., 2011). Microarray analysis of NrleGFP retina revealed that a novel cell surface antigen CD24 was also expressed by developing mouse retina (Lakowski et al., 2011). Indeed, FACs sorting cells from the developing mouse retina based on the expression of CD24 and CD73 proved to be a powerful purification strategy. Transplantation of cells sorted based on the CD73/CD24 in the subretial space of wild type mouse retina revealed an 18- and 2.3-fold increase in integration in the ONL compared to unsorted and NrlEGFP sorted cells respectively.

Purification of hESCs- or iPSCs- derived retinal cells based on these cell surface antigens and their subsequent transplantation into animal models of photoreceptor dystrophies would be a milestone achievement and one huge step towards hESCs- and iPSCs- based human retinal cell therapy.

1.4. Low oxygen (O_2) on human pluripotent stem cell differentiation

1.4.1. O_2 tension during early mammalian development and hypoxia-inducible factors (HIFs)

During the early stages of mammalian development, embryos develop in the well- controlled environment of the uterus (Okazai and Maltepe, 2006). Among the important components, O_2 tension has been reported to play a critical role on the embryonic development (Dunwoodie 2009). The uterus environment is considered to be hypoxic, as the O_2 tension during the embryonic development is considerably lower than the atmospheric O_2 tension (~20%) (Lee et al., 2001; Pringle et al., 2007). For instance, the human embryo develops in an environment where O_2 tension ranges between 0 and ~3% until the start of the second trimester (Burton and Jaunaiux, 2001) and other mammalian embryos experience a similar deficiency in the supply of O_2 during the early developmental stages (e.g. rabbit 8.7% and monkey 1.5%) (Fischer and Bavister, 1993). Hypoxia seems to play a critical role in organogenesis as cells found in the heart, gut, lung, midbrain, pituitary, spinal cord and tongue of the E14.5 mouse embryo stained positive for a hypoxic marker Primonidazole (Dunwoodie, 2009). This was further supported when the exposure of the developing embryos to the atmospheric level was found to be detrimental to the development of key organs which led to embryonic lethality (Catt and Henman, 2000).

In hypoxic conditions, the stabilization of hypoxia-inducible factor-1a (HIF-1a) is a key feature of the cellular response (Wang et al., 1995). HIF-1a is subject to a proteasomal degradation under the atmospheric O_2 but upon stabilization in hypoxic conditions, HIF-1α dimerizes with HIF-1β and the dimer binds to the hypoxic response element (HRE) to transactivate some 250 HIF target genes (Huang et al., 1996; Kallio et al., 1997). These genes are thought to be responsible for various cellular activities required for the adaption in the oxygen-deficient environment such as angiogenesis, erythropoiesis, energy metabolism and cell survival (Ke and Costa, 2006). The activation of these however, seems unlikely to take place at the same time when cells are exposed to hypoxia and it is more plausible that the transcriptional responses are context- and cell type- dependent. It has also been reported that stabilized HIFs can also function in a non-canonical pathway by a cross-talk with other signal pathways. Gustafsson et al extensively studied the interaction between HIF-1a and Notch signal pathway (Zheng et al., 2008). Notch pathway is conserved across different species and plays important roles during development (Schweisguth, 2004). Notch pathway is activated when a ligand binds to the transmembrane Notch receptor which releases intracellular domain (ICD). ICD then translocates to the nucleus and activates downstream target genes of Notch such as Hes and Hey genes. The activation of Hes1 and Hey2 is enhanced

under hypoxic condition and it was revealed, by immunoprecipitation, that there is a physical interaction between HIF-1a and Notch 1 ICD (Gustafsson et al., 2005). It is thought that HIF-1a stabilizes Notch ICD to enhance its transcriptional activity.

1.4.2. Hypoxia and neuronal development

The stabilisation of HIF has been shown to be critical for the survival of the embryos and during organogenesis. By generating HIF-deficient mice, studies have demonstrated that embryos were morphologically abnormal as early as E8.5 and caused embryonic lethality by E10.5 (Ramirez-Bergeron et al., 2006). The histological analysis of embryos at E9.75 revealed defects in the development of vascularization such as dilated vascular structures and reduction in the overall number of cephalic blood. The involvement of HIF in the development of vascular structure has been studied in depth however, its role in the development of CNS remains unclear. By creating neural specific HIF-knockout mice, it has been demonstrated that HIF plays a critical role in the normal development of functional brain (Tomita et al., 2003). The mutant mice, although showed no external differences, had impaired spatial memory functions and a significantly reduced number of neuronal cells.

The effect of hypoxia on neuronal developmental has been studied with many interests over the last 10 years. Cells from various parts of the brain were isolated and subject to hypoxic conditioning in an attempt to elucidate the effect of hypoxia on neurogenesis. Lowering O_2 tension during the culture of ventral midbrain cells enhanced the generation of tyrosine-hydroxlase (TH) positive dopaminergic neurons across the different species (Milosevic et al., 2005; Studer et al., 2000).

Human neural crest stem cells (NCSCs) which have the capacity to differentiate into autonomic neurons in response to BMP exhibited an enhanced survival, proliferation and multilieange differentiation when cultured under 5% O_2 (Morrison et al., 2000). Mesencephalic precursors derived from human fetal mesencephalon are capable of differentiating into dopamine neurons (DA). The O_2 level of Interstitial tissue in the mammalian brain ranges between 0.55 and 8% (Erecinska and Silver, 2001). It has been demonstrated that mesencephalic precursors' proliferative capacity is significantly enhance when cultured at 3% O_2 in the presence of epidermal growth factors (EGF) and bGFG2 (Storch et al., 2001). In addition, it was observed that differentiating precursors derived from human fetal midbrain in the presence of brain derived neurotrophic factor (BDNF) and ascorbic acid for 21 days at 5% O_2 resulted in up to 50% of total cells expressing MAP2- and TH- positive DA (Maciaczyk et al., 2008). Human neural stem cells (hNSCs) isolated from diencephalic and telecephalic brain regions of fetus at 10.5 week gestational age showed an improved survival, proliferation and maintenance of an undifferentiated phenotype when cultured at 5% O_2 tension (Santilli et al., 2010).

Amongst 250 downstream targets of HIF, vascular endothelial growth factor (VEGF) is a well-known growth factor for the formation and maintenance of vascular structure (Marti and Risau, 1998; Shweiki et al., 1992). However, studies

have demonstrated that VEGF plays pleiotropic roles in promoting cellular growth, proliferation and protection in the CNS (Sanchez et al., 2010). The neurotrophic role of VEGF has been demonstrated when VEGF promoted neurite outgrowth and enhanced survival of dopaminergic neurons when applied to the fetal ventral mesencephalic explants (Jin et al., 2000; Rosenstein et al., 2003; Sondell et al., 2000). In addition, VEGF induces neurite growth and is a potent mitogen for astroglia and Schwann cells as well as mediates mouse RPCs proliferation in-vitro through VEGF receptor during mouse embryogenesis (Sondell et al., 1999; Yang and Cepko, 1996). Erythropoietin (EPO) is another downstream target of HIF-1a, widely known for its roles in the production of red blood cells (Semenza et al., 1991). The expression of EPO receptors in the developing mouse and human CNS supports a possible role for non-hematopoeitic functions of EPO. Indeed, recent studies have reported that EPO is required for normal brain development and its neurotrophic activity in the CNS is regulated by the O_2 tension and tissue hypoxia (Juul et al., 1999; Yu et al., 2002). To support this, Pistollato et al demonstrated an enhanced proliferation of human CNS precursor cells at 5% compared to 20% which are accompanied by the expression of erythropoietin EPO that is required for neural precursor survival and expansion *in vivo* (Pistollato et al., 2007).

1.4.3 Hypoxia on pluripotency and differentiation of hESCs

Investigation of the effect of low oxygen on hESCs biology has attracted much attention in the last decade. Perhaps this comes as no surprise as all cells in the embryo are exposed to low oxygen and also differentiate during organogenesis at a reduced level of oxygen (see section 1.4.1). This presents oxygen tension as an important variable for many aspects of hESCs studies. The effect of hypoxia on the way hESCs behave is a context-dependent. Exposing cells kept in the self-renewing condition to lowered O_2 tension revealed a better maintenance of pluripotency compared to cells exposed to 20% O_2 (Ezashi et al., 2005; Forsyth et al., 2006; Zachar et al., 2010). Hypoxia seemed not to alter the expression of pluripontency genes directly, but transcriptomic analysis revealed widespread differences in the expression of pluripotency target genes between hypoxic and normoxic conditions (Forsyth et al., 2008; Westfall et al., 2008). Most notably, the upregulation of Lefty2 was observed which is thought to prevent spontaneous differentiation of hESCs. Indeed, it was revealed by an independent study, that a greater heterogeneity was observed in cultures at 20% O_2 compared to 5% culture (Ezashi et al., 2005). This heterogeneity could arise from subpopulations of colonies that may have already begun to differentiate at 20% O_2.

Numerous studies have attempted to investigate whether hESCs differentiation could be enhanced by exposing differentiating hESCs to the

physiological oxygen tension that the developing embryo experiences. Previously, effect of hypoxia on neuronal differentiation of mouse ESCs was investigated (Mondragon-Teran et al., 2009). The results revealed that 2% O_2 failed to show any differences in the production of NSCs expressing Nestin. However, their subsequent differentiation into neuronal cells expressing bIII tubulin and MAP2 was significantly enhanced compared to the differentiation at 20% O_2. In a similar study, mESCs were differentiated into NSCs under 20% O_2 and their subsequent differentiation into oligodendrocyte was enhanced at 3.5% compared to 20% O_2 as more DA cells expressing TH (Kim et al., 2008). This trend was also seen with neuronal differentiation of hESCs. Stacpoole et al (2011) demonstrated that neural conversion of hESCs to Nestin-expressing NSCs is not affected by lowering O_2 tension (3% O_2). These NSCs were then directed to differentiate into midbrain dopaminergic and spinal motor neurons by adding FGF-8 for 1 week and FGF-8 with 1mM purmorphamine for 1–2 weeks. qPCR analysis revealed that NSCs directed to differentiate at 3% O_2 had a two- and five-fold increase in the expression level of OLIG2 and EN-1 respectively, both specific markers for motor neuron and midbrain dopaminergic neuron precursors (Stacpoole et al., 2011). Other lineage differentiations that have been reported to benefit from operating at a low O_2 tension include chondrogenic and endothelial differentiation of hESCs and cardiac

differentiation of mESCs (Koay and Athanasion, 2008; Bianco et al 2009; Prado-Lopez et al., 2010).

1.5. Summary

Human pluripotent stem cells have the potential to differentiate into almost all types of cells in the body whilst maintaining the ability to self-renew (Section 1.2.1 and 1.2.2). These unique properties are ideal for providing a platform for the development of a novel cell replacement therapy for treatment of currently incurable degenerative conditions. Retinitis pigmentosa (RP) is one example of a genetic condition that could benefit if the potential is to be realised (Section 1.1.2, table 1.1). Patients suffering from RP experience visual impairment due to degeneration of photoreceptors in the retina (Hartong et al., 2006). The condition remains untreatable however, recent studies have shown that clinically-viable retinal cells could be produced from human pluripotent stem cells (Lamba et al., 2010 and Section 1.3.1). Despite this promising development, clinical application of human pluripotent stem cells suffers from major drawbacks. Along with safety concerns, a consistent large scale production of specialized cells from human pluripotent stem cells remains a major hurdle to successful therapy (Gerecht-Nir and Itskovitz-Eldor, 2004; Oh and Choo, 2006). In order to develop a robust process for producing enough transplantable cells from human pluripotent stem cells for therapeutic purposes, it is important to optimize current differentiation protocols. They rely on the addition of growth factors that have been identified to play key roles in tissue development (Section 1.2.2). However, as described previously in Section 1.3, current

differentiation protocols are not only inefficient and lengthy, but their efficiency varies depending on the human pluripotent stem cells lines used.

A few recent studies have demonstrated that mimicking the microenvironmental oxygen (O_2) tension of the developing mammalian embryo could significantly increases pluripotent stem cell differentiation efficiencies into various lineages of cells (Section 1.4.3). The O_2 concentration in the uterine environment ranges between 1 and 5% which is significantly lower than the atmospheric concentration (21%, see section 1.4.1). This is also the environment where organogenesis takes place therefore mimicking this O_2 tension could have a significant impact on differentiation.

1.6 Hypothesis

The hypothesis of this thesis is that mimicking physiological O_2 tension enhances the generation of RPCs from human iPSCs and hESCs. In order to test the hypothesis, the study was divided into three major sections.

Characterisation of hESCs and iPSCs. Chapter 3 characterizes two different human pluripotent stem cell lines used for this study. Characterisation is based on the expression of typical pluripotency genes, their ability to differentiate into three germ layer and karyotypic analysis.

Retinal differentiation capacity of iPSCs and hESCs. Chapter 4 next compares production of RPCs from iPSCs and hESCs using an established differentiation protocol (Lamba et al., 2006). This is important to provide the control data against which any benefits of operation under hypoxic conditions can be quantified.

Optimisation of RPC generation from hESCs and iPSCs. Chapter 5 investigates the role environmental oxygen plays in optimising retinal differentiation protocol. This is assessed in terms of the number and purity of cells expressing RPC markers.

In addition to the literature survey describing in this chapter, Chapter 2 covers all the materials and methods used during the investigation while Chapter 6 summarise the main conclusions and also provides suggestions for future work.

2.Materials and Methods

2.1 Human Pluripotent Cell Culture

Undifferentiated human pluripotent cells (MSUH001 iPSCs and Shef3 hESCs were obtained from the Spanish and the UK Stem Cell Bank respectively), were grown on Mitomycin-C (1mg/ml, Sigma-Aldrich, Poole, UK) inactivated mouse embryo fibroblasts (MEF) in Knockout DMEM supplemented with 20% (v/v) Knockout serum replacement, 1mM L-glutamine, 1%(v/v) non-essential amino acids, 100mM β-mercaptoethanol, and 4ng/mL bFGF (all Invitrogen, Paisley, UK). They were cultured in a Sanyo IncuSafe incubator (Sanyo, MCO-18AIC, Leicestershire, UK) at 37℃ and 5%(v/v) CO_2 and 5ml of fresh growth medium was exchanged every 24 hours. iPSCs were transferred to newly Mitomycin-C inactivated MEF every 3-4 days by dissecting colonies into smaller clumps with Fine Tip Mini Pastette (Alpha Laboratories, Hampshire, UK). hESCs colonies were dissected in the same way after incubating with 0.025mg/ml collagenase (Invitrogen) for 3 minutes at 37℃.

2.2 Isolation of MEF

Mouse embryos from the female at day 13 p.c. were obtained. Each embryo was separated from the placenta and surrounding membranes using sterile tweezers.

The embryo sac was cut open using a fine surgical scissors and the brain and dark organs were removed. The embryos were collected in PBS and finely minced before being incubated wit 2ml of Trypsin (Invitrogen) at 37℃ for 15 minutes. The supernatant was collected and resuspended in 2ml MEF medium that consists of DMEM supplemented with 10%(v/v) Foetal bovine serum (all from Invitrogen) and 1%(v/v) NEAA (Gibco). This was subjected to a low speed centrifugation (1200rpm) for 3 minutes. The supernatant was removed and the pallet was resuspended in MEF before being plated in a T75 flask (Nunc) denoted as "P0".

2.3 Feeder preparation

When MEF reached between 70 and 80% confluency, they were inactivated with 9ml pre-diluted Mytomycin-C for 2 hours at 37ºC. The cells were then washed three times with 5ml DPBS and trypsinized for three minutes at 37ºC. The cells were then quenched with 3ml MEF medium and centrifuged at 1200rpm for 3 minutes. The supernatant was removed and the pallet was resuspended in 20ml MEF medium for cell counting before plating 250,000 cells/cm² in a T25 flask.

2.4 Three germ layer differentiation

Human pluripotent stem cell colonies were removed from the feeder layers

and cultured in suspension for 15 days in the hES medium without bFGF. The subsequent EBs were subjected to immunocytochemistry in suspension as described in section 2.7. The list of primary and their corresponding secondary antibodies can be found in Table 2.1. The expression of the markers was observed with confocal microscopy (Perkin Elmer Ultraview Spinning Disk).

2.5 Early Neural Retinal Differentiation of Human pluripotent Stem Cells

Human pluripotent stem cells were induced to differentiate using a previously published protocol (Lamba et al., 2006). Briefly, undifferentiated pluripotent stem cell colonies were dissected into small clumps with Fine Tip Mini Pastette (Alpha Laboratories) and cultured as embryoid bodies (EBs) in suspension in 30mm non-adherent bacterial grade culture dishes (Sterilin, Caerphilly, UK) containing 3ml retinal induction medium consisting of DMEM/F12 supplemented with 10%(v/v) Knockout Serum Replacement (both Invitrogen), 1ng/ml human recombinant DKK-1, 1ng/ml human recombinant noggin, 5ng/ml human recombinant IGF-1 (all R&D Systems, Minneapolis, US) and 1%(v/v) N-2 Supplement (PAA Laboratories Ltd, Yeovil, UK) for 3 days (Fig 2D). On day 4, approximately 30 EBs were selected under a dissecting microscope and plated in each well of a 6-well plate coated with Matrigel (BD Bioscience, San Diego, CA)

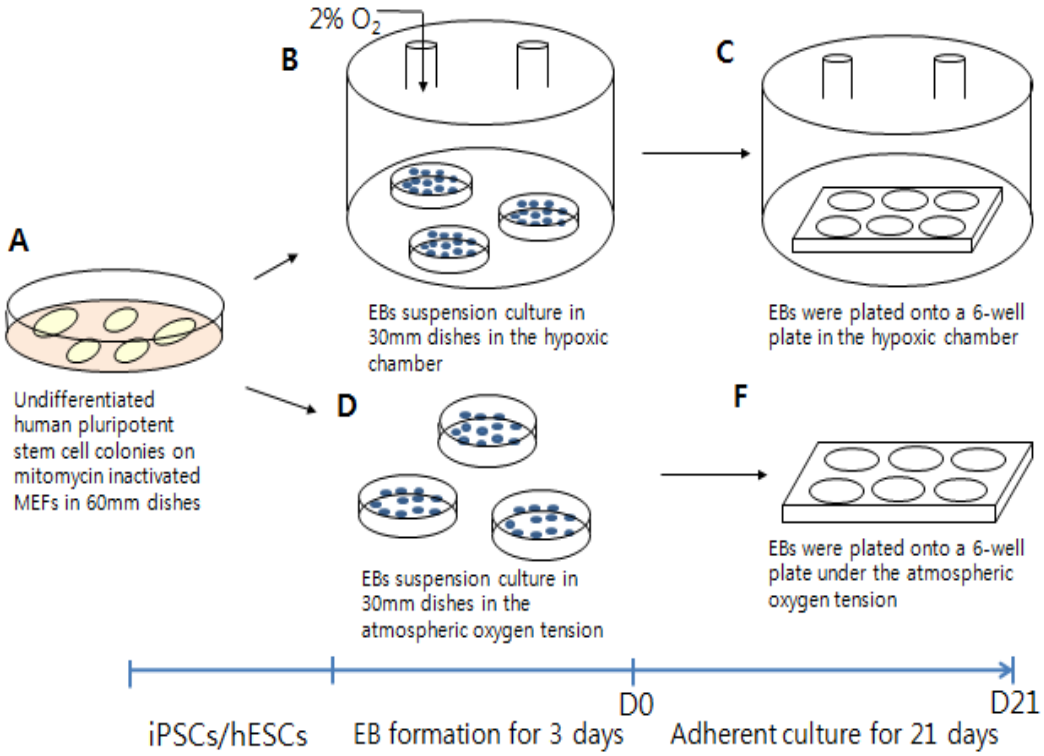
and cultured in DMEM/F12 (Invitrogen) supplemented with 10ng/ml human recombinant DKK-1, 10 ng/ml human recombinant IGF-1, 10ng/ml human recombinant Noggin, 5ng/ml basic fibroblast growth factor (all R&D Systems), 1%(v/v) N-2 Supplement and 2%(v/v) B27 Supplement (PAA Laboratories Ltd) (Fig 2.1F).

2.6 Oxygen Tension Control

The hypoxic chambers used in these studies had previously been fabricated to maintain 2% O_2 during the neuronal differentiation mESCs (full description of construction and operation can be found in (Mondragon-Teran et al., 2009)). Briefly, undifferentiated pluripotent stem cell colonies were dissected into small clumps as described above before placing the cell culture dish into a hypoxic chamber. To maintain humidity within the chamber, a 30 mm petri dish (Sterilin, Caerphilly, UK) containing 3ml of distilled water was also placed in the chamber. The chamber was then gassed from a premixed gas cylinder composed of 2%(v/v) O_2, 5%(v/v) CO_2 and 93%(v/v) N_2 (BOC, Barking, UK) for 5 minutes before placing it in an incubator at 37℃ (Fig 2B). After 3 days, the culture dishes were removed from the chamber and plated into 6-well plates coated with Matrigel as described above before being returned to the chamber, gassed and placed back into the incubator (Fig 2C). The

medium was changed every 2 days during which the chamber was re-gassed.

Figure 2



Growth factors	bFGF	DKK-1(1ng/ml), Noggin (1ng/ml), IGF-1 (5ng/ml)	DKK-1 (10ng/ml), Noggin (10ng/ml), IGF-1(10ng/ml), bFGF (5ng/ml)
Analysis	FACS, Immunocytochemistry	qPCR, Immunocytochemistry	qPCR, Immunocytochemistry, FACS

Figure 2. Schematics of retinal differentiation of human pluripotent stem cells. (A) Human pluripotent stem cells were cultured in the presence of MEF to maintain pluripotency in the ambient O_2 tension. (B) Colonies were removed from the feeder layer and cultured in suspension to form EBs for 3 days in the hypoxic chamber where 2% O_2 was maintained. (C) After 3 days, subsequent EBs were plated on matrigel-caoted 6-well plate, again both in the hypoxic chamber and incubator. (D and E) EBs were also made in the normoxic conditions for 3 days and plated on a matrigel-coated 6-well plate.

2.7 Immunocytochemistry

Cells were fixed with 2ml 4%(w/v) paraformaldehyde (PFA) for 20 minutes before being permeabilized with 0.25%(v/v) Triton X-100 in Dulbecco's modified phosphate-buffered saline (PBS) for 10 minutes at room temperature (all from Sigma-Aldrich). The samples were washed with 2ml DPBS and incubated in 2ml blocking solution (2%(v/v) goat serum, 0.25%(v/v) Triton X-100 in PBS all from Sigma-Aldrich) for 30 minutes. For the detection of cell surface marker, the permeabilization step was omitted and cells were incubated straight into the blocking solution for 30 minutes. The samples were then incubated with 600μl of primary antibodies diluted in blocking solution overnight at 4℃. The primary antibodies used were as follows; monoclonal mouse anti-Oct4 IgG (Invitrogen 1:400), monoclonal mouse anti-Tra-1-60 IgM (1:200), monoclonal mouse anti-SSEA3 IgM (1:200), polyclonal mouse anti-Nestin IgG (1:400), monoclonal mouse anti-Pax6 IgG (1:300), polyclonal rabbit anti-Otx2 (1:1000), monoclonal mouse-Neurofilament-M IgG (1:400), polyclonal rabbit anti-S-opsin (1:200), polyclonal rabbit anti-Nrl (1:200, all from Millipore, Hertfordshire, UK), polyclonal Rabbit anti-Chx10 IgG (Sigma-Aldrich, 1:300), monoclonal mouse anti-rhodopsin (1:200), monoclonal mouse anti-Prox1 (1:200), polyclonal goat anti-IRBP (1:200), polyclonal goat anti-Six3 (1:200), polyclonal goat anti-Lhx2 (1:200, all from Santa Cruz), monoclonal mouse anti-Crx (Abnova), monoclonal rabbit-anti-PKCα IgG (1:400, Abcam). The cells were then

washed with 2ml PBS three times and incubated with 600µl of either Alexa Flour 488 goat anti-mouse, Alexa Flour 488 goat anti-rat IgM, Alexa Flour 555 goat anti-mouse (all Invitrogen, 1:400), and Cy3 conjugated donkey anti-rabbit IgG (Millipore, 1:300) in blocking solution for 1 hour at room temperature (details of primary and secondary antibody combinations can be found in Table 2.1). The same concentration of the corresponding isotype controls were used as a negative control (murine IgM isotype control (Sigma-aldrich), mouse and rabbit IgG isotype control (both from Abcam)).The cells were washed three times with 2ml PBS and incubated with 4,6-diamidino-2-phenylindole (DAPI, Invitrogen, 1:1000) in PBS for 5 minutes. Fluorescence images were acquired using a fluorescence microscope (Nikon, Eclipse TE2000-U) and analyzed with NIS-element software. Confocal images were taken with Perkin Elmer Ultraview Spinning Disk confocal microscope.

Table 2.1

Samples	Primary antibody (dilution)	Secondary antibody (1:400)
MSUH001-iPSCs	Mouse anti-Oct4 (1:400)	Goat anti-mouse Alexa Flour 488
	Rat anti-Tra-10 (1:200)	Goat anti-rat Alexa Flour 488
	Mouse anti-SSEA3 (1:200)	Goat anti-mouse Alexa Flour 488
Shef3-hESCs	Mouse anti-Oct4 (1:400)	Goat anti-mouse Alexa Flour 555
	Mouse anti-Tra-1-60 (1:200)	Goat anti-mouse Alexa Flour 555
iPSCs- and hESCs-EBs	Mouse anti-Oct4 (1:400)	Goat anti-mouse Alexa Flour 488
hESCs- and iPSCs- RPCs	Rabbit anti-Otx2 (1:1000)	Goat anti-rabbit Alexa Cy3
	Mouse anti-Lhx2 (1:200)	Goat anti-mouse Alexa Flour488
	Mouse anti-Six3 (1:200)	Goat anti-mouse Alexa Flour488
	Mouse anti-Pax6 (1:400)	Goat anti-mouse Alexa Flour 488
	Rabbit anti-Chx10 (1:300)	Goat anti-rabbit Cy3
	Mouse anti-Crx (1:200)	Goat anti- mouse Alexa Flour 555
	Rabbit anti-Nrl (1:400)	Goat anti-rabbit cy3
	Goat anti-IRBP (1:200)	Donkey anti-goat Alexa Flour 488
	Rabbit anti-S-opsin (1:200)	Goat anti-rabbit Cy3
	Mouse anti-Rhodopsin (1:200)	Goat anti-mouse Alexa Flour 488
	Mouse anti-Neurofilament-M (1:200)	Goat anti-mouse Alexa Flour 488
	Mouse anti-Prxo1 (1:200)	Goat anti-mouse Alexa Flour488
	Rabbit anti-PKCα (1:400)	Goat anti-rabbit Cy3

Table 2.1. The list of primary antibodies used and their corresponding secondary antibodies.

2.8 Detection of Pimonidazole Hydrochloride at 2% O2

The hypoxia marker pimonidazole hydrochloride (Hypoxyprobe-1 kit, HPI Inc., MA, USA) was used to demonstrate the exposure of cells to lowered O_2 tension in the hypoxic chamber. iPSCs and hESCs were cultured at both 20% and 2% O_2 for two days. 100μM pimonidazole was added two hours prior to fixation with 4% PFA. The fixed samples were blocked as previously described and incubated with anti-pimonidazole mouse monoclonal antibody IgG1 (HPI Inc., 1:30 in PBS) or mouse IgG isotype control (Abcam) overnight at 4℃. The samples were washed with 1ml PBS and incubated with 600μl of Alexa Flour 488 goat anti-mouse IgG (Invitrogen 1:400) for two hours at room temperature. The samples were washed with 1ml PBS and incubated with DAPI (Invitrogen 1:1000) for two minutes in the dark before analysed by confocal microscopy.

2.9 Flow Cytometry

2.9.1 Cell surface markers

Samples were washed with 2ml DPBS and incubated with 2ml Trysin (Invitrogen) for 10 minutes at 37℃. The cultures were quenched with DMEM containing 10% (v/v) FBS (Invitrogen) and physically dissociated by pupating 10 times with a Pasteur pipette (Fisher Scientific, Leicestershire, UK). The dissociated cells were re-suspended in DMEM containing 10% (v/v) FBS and centrifuged for 3

minutes at 1200 rpm. The supernatant was removed and the pellet was re-suspended in 2ml of PBS. After centrifuging at the same conditions, the supernatant was removed and the cells were fixed with 0.5ml 4%(w/v) PFA for 20 minutes on ice. The cells were centrifuged at 1200rpm for 3 minutes and re-suspended in 0.5ml blocking solution (2%(v/v) goat serum in DPBS) for 30 minutes on ice. The cells were again centrifuged at the same condition and incubated with the blocking solution containing monoclonal mouse anti-Tra-1-60 IgM (1:200) and murine IgM isotope control (Sigma-Aldrich, 1:200) overnight at 4℃. The cells were washed twice with 1.5ml DPBS and incubated in blocking solution containing Alexa Flour 488 goat anti-mouse IgM (Invitrogen) for two hours at room temperature. The cells were washed twice with 1.5ml DPBS before incubating with DAPI for three minutes. FACs analysis was then performed using a flow cytometer (CyAN ADP, 9 colour, 3 laser analyser, Beckman Coulter, High Wycombe, UK). Dead cells and debris were excluded using forward and side scatter parameters and only single cell population was selected using the pulse width parameter. 99% of samples incubated with the isotype control and a secondary antibody was gated as a negative population. The data were analysed with Sumit v4.3 software (DAKO, Cambridgeshire, UK).

2.9.2 Intracellular markers

After fixing cell with PFA, the cells were washed with DPBS and incubated with 0.5ml permeabilizing solution (0.25% Triton in DPBS) on ice for 10 minutes. After centrifugation, the cells were blocked in 0.5ml DPBS with 2%(v/v) goat serum, 0.25%(v/v) triton for 30 minutes on ice before incubating overnight at 4°C with blocking solution containing murine monoclonal IgM isotope control (1:200), monoclonal mouse anti-Oct4 IgG (1:400), monoclonal mouse anti-Pax6 IgG (1:300) and polyclonal Rabbit anti-Chx10 IgG (1:300). The cells were subjected to three DPBS washes before incubating with blocking solution containing Alexa Flour 488 goat anti-mouse IgG and Alexa Flour 647 goat anti-rabbit IgG (Invitrogen) for two hours at room temperature. Isotype controls were used (same concentration as the primary antibodies) as a negative control (mouse and rabbit IgG isotype control (both from Abcam)). The cells were washed, counter-stained with DAPI and analysed as described above.

2.10 RNA extraction and cDNA synthesis

Total RNA extraction was achieved using the Qiagen RNeasy kit (Qiagen, Crawley, UK). Briefly, cell pellets were harvested using a cell scraper (TPP Techno Plastic Products AG, Switzerland), re-suspended and homogenized by aspirating 5 times through an RNase-free syringe with an 18G needle in buffer RLT provided in

the kit. The samples were then processed in accordance with the manufacturer's instructions. In the final step, RNA was eluted with 40µl of RNase-free water by centrifuging for 1 minute at 10,000 rpm. The RNA concentration was determined by measuring absorbance at 260 nm using a spectrophotometer (NanoDrop ND-1000, Thermo Scientific, Epson, UK). For cDNA synthesis, we used the Ambion 1st strand cDNA synthesis kit (RETROscript® Ambion, Warrington, UK) according to manufacturer's instructions with 1µg of RNA for each reaction in a total reaction volume of 20µl.

2.11 Real-time quantitative polymerase chain reaction (qPCR)

qPCRs were performed using the MESA BLUE qPCR MasterMix Plus for SYBR® Assay (Eurogentec, Hampshire, UK) following the manufacturer's instructions. All primer pairs were acquired from Qiagen which are listed in Table 2.2. Each reaction contained 1µL of 1st strand cDNA in a total reaction volume of 20µL and all measurements were taken in triplicate. The PCR conditions used were as follows; 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles at 95°C for 15 seconds, 55°C for 30 seconds and 60°C for 30 seconds. The final three steps were 95°C for 15 seconds, 60°C for 15 seconds and 95°C for 15 seconds. All samples were normalized to levels of β-actin (an endogenous housekeeping gene) and relative

quantification was carried out as previously described (Pfaffl, 2001).

Table 2.2

Official Symbol	Official name	Detected transcript	Catalogue number
ACTB	Actin, beta	NM001101	QT01680476
OTX2	Orthodenticle homeobox 2	NM 021728	QT00213129
LHX2	LIM homeobox 2	NM 004789	QT00065359
SIX3	SIX homeobox 3	NM 005413	QT00211897
PAX6	Paired box 6	NM 000280	QT00071169
VSX2 (CHX10)	Visual system homeobox 2	NM 182894	QT00221081
RAX(RX)	Retina and anterior neural fold homeobox	NM 013435	QT00212667
CRX	Cone-rod homeobox	NM 000554	QT01192632
NRL	Neural retina leucine zipper	NM 006177	QT01005165
RHO	Rhodopsin	NM 000539	QT00035700
OPN1SW	Opsin 1 (cone pigments), short-wave-sensitive	NM 001708	QT00017304

Table 2.2. The list of primers used for qPCR analysis. All the primers were acquired from Qiagen.

2. 12 Statistical Analysis

Triplicate samples were analysed in all experiments. The values are expressed as means ± standard error of the mean (S.E.M). To determine statistical significance, Paired Student's T-tests were performed and $P \leq 0.05$ was considered significant.

3. Characterisation of MSUH001-iPSCs and Shef3-hESCs

3.1. Introduction

Human pluripotent and embryonic stem cells are characterized by their ability to maintain (i) prolonged undifferentiated proliferation and (ii) developmental potential to form derivatives of all three embryonic germ layers (Thomson et al., 1998). These unique properties are ideal for the development of novel cell replacement therapies for a numerous degenerative medical conditions (Oliveri and Anderson, 2008). Various laboratorial techniques are available to examine whether cells fulfill the criteria to be considered as pluripotent stem cells. Stage-specific embryonic antigens (SSEA)-3, SSEA-4, Tra-1-60 and Tra-1-81 are the classic cell surface markers that are present in undifferentiated cells (Carpenter et al, 2003). Immunocytochemistry or flow cytometry using antibodies raised against these markers is the commonly used method to characterize undifferentiated state of pluripotent stem cells (Thomson et al., 1998). In addition, the same technique can be performed with antibodies raised against pluripotency-associated transcription factors such as Oct4, Nanog and Sox2 (Loh et al., 2006).

Multi-lineage differentiation capacity of pluripotent stem cells can be demonstrated both *in vitro* and *in vivo*. Pluripotent stem cells spontaneously differentiate into cells representing three germ layers through EB formation *in vitro*

(Thomson et al., 1998; Reubinoff et al., 2000). EBs are formed when colonies are removed from the feeder layers and cultured in suspension without any growth factors. They form spheroid aggregates of cells that contain cells of all the three germ layers in a disorganized fashion (Chen et al., 2010). Transplanting pluripotent stem cells in immunodeficient (SCID) mice forms a tumour-like structure termed teratoma. Histological examination of teratoma reveals that it contained various tissues including cartilage (mesoderm), gut-like epithelial cells (endoderm) and neural tissues (ectoderm) (Thomson et al., 1998; Takahashi et al., 2007). Another *in vivo* method for the test of pluripotency is the generation of chimera. This method examines whether pluripotent stem cells contribute to the development of all adult tissues when injected into a recipient blastocyst (Evans and Kaufman, 1981). Due to ethical reason however, this technique cannot be carried out with primate ES cells.

3.1.1. Aim

The aim of this chapter is to examine whether human induced pluripotent stem cell (MSUH001-iPSCs) and embryonic stem cell (Shef3-hESCs) lines fulfilled the essential criteria of pluripotent stem cells.

3.2. Characterization of MSUH001-iPSCs

3.2.1. Morphology and undifferentiated state

The first human iPSCs lines were created by two independent groups in 2007 (Takahashi et al., 2007; Yu et al., 2007). They genetically reprogrammed human somatic cells by forcefully expressing a combination of key pluripotency genes using viral transfection systems. iPSCs have revolutionized stem cell research due to their potential to create patient- and disease-specific pluripotent stem cells for cell replacement therapy (Nishikawa et al., 2008). In addition, they could provide a useful tool for understanding disease mechanism, drug screening and toxicity testing (Heng et al., 2009; Marchetto et al., 2011). In this experiment, MSUH001 (P11) iPSCs line was used, which had previously been generated in Jose Cibelli's lab (Michigan State University) by expressing a combination of four factors (Oct4, Sox2, Nanog and Lin28) in human somatic fibroblasts (Yu et al., 2007). These cells were cultured in the presence of Mytomycin-C inactivated MEF feeder cells and grew as colonies of cells. After 5 passages, it was revealed that, although colonies were flat and round which are typical characteristic of human pluripotent stem cell colonies (Reubinoff et al., 2000), the culture contained a heterogeneous population of colonies based on their morphologies (Fig 3.1A). Further analysis with immunocytochemistry revealed that the culture contained three sub-populations of colonies based on the expression of Oct4 (Fig 3.1B). We identified colonies a type I sub-population that did

not express Oct4 (Fig 3.1B top row), a type II sub-population that partially expressed Oct4 (Fig 3.1B middle row) and a type III sub-population that expressed Oct4 (Fig 3.1B bottom row). In order to obtain a homogeneous population of true pluripotent iPSCs colonies, colonies that morphologically belonged to the type III sub-population were selectively passased (Fig 3.1B bottom row). Morphological analysis by phase contrast microscopy after 7 passages revealed that the most of colonies were round and flat, consisting of single cells with a high nucleus/cytoplasm ratio (Fig 3.2A). Immunocytochemistry confirmed that all the colonies expressed pluripotency and undifferentiated markers Oct4 and Tra-1-60 in the nucleus and membrane respectively (Fig 3.2C). Flow cytometry showed a high percentage of the population expressed Oct4 (92±1.56%) and Tra-1-60 (84±2.05%) (Fig 3.3A and B). This indicates that cells maintained undifferentiated state whilst they were propagated in culture.

Figure 3.1

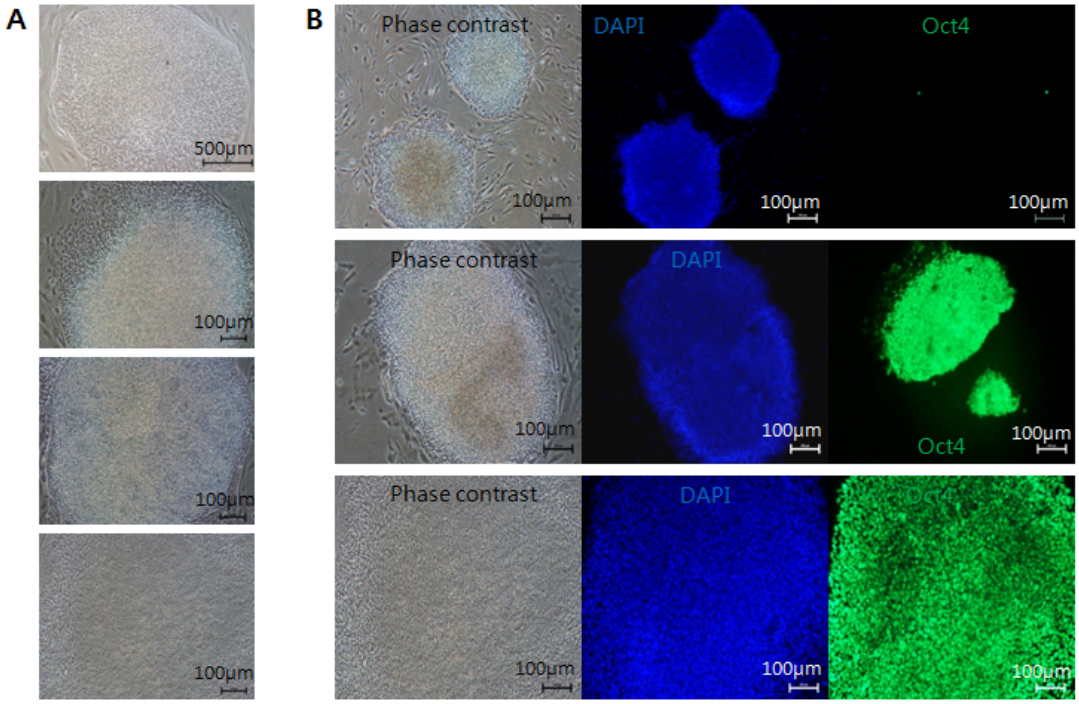


Figure 3.1. Heterogeneous population of MSUH001-iPSCs culture. (A) Morphologically different colonies were present in the culture after 5 passages from thawing P11 MSUH001-iPSCs. (B) Three sub-populations of colonies were identified based on Oct4 expression. They were colonies that did not express Oct4 (top panel), colonies that partially expressed Oct4 (middle panel) and colonies that expressed Oct4 (bottom panel).

Figure 3.2

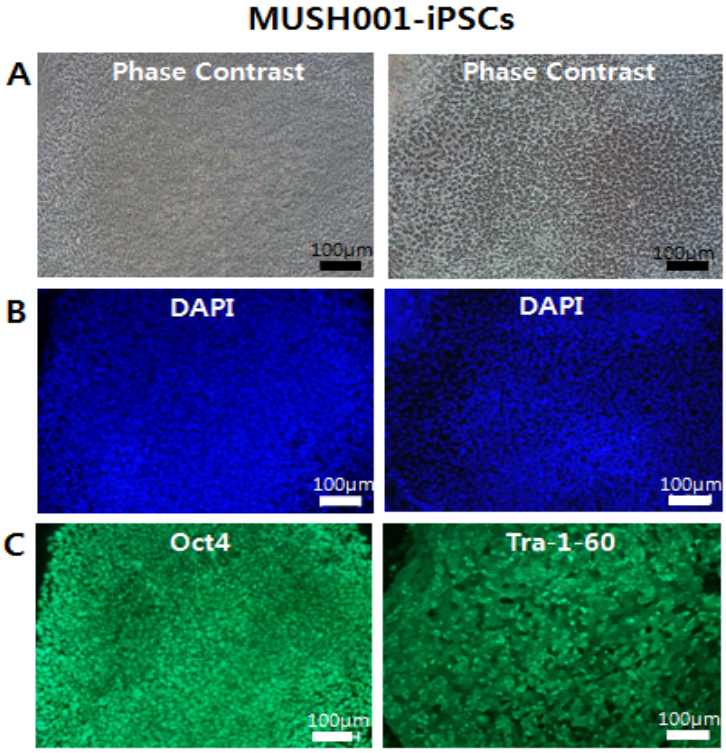


Figure 3.2 Characterization of MSUH001-iPSCs. (A) iPSCs were cultured in the presence of MEF feeder layer to maintain pluripotency. They exhibited round and compact colonies, consisted of tightly packed single cells. (B) DAPI counter-staining for nucleus. (C) The colonies expressed undifferentiated stem cell makers Oct4 and Tra-1-60 in the nucleus and membrane respectively

Figure 3.3

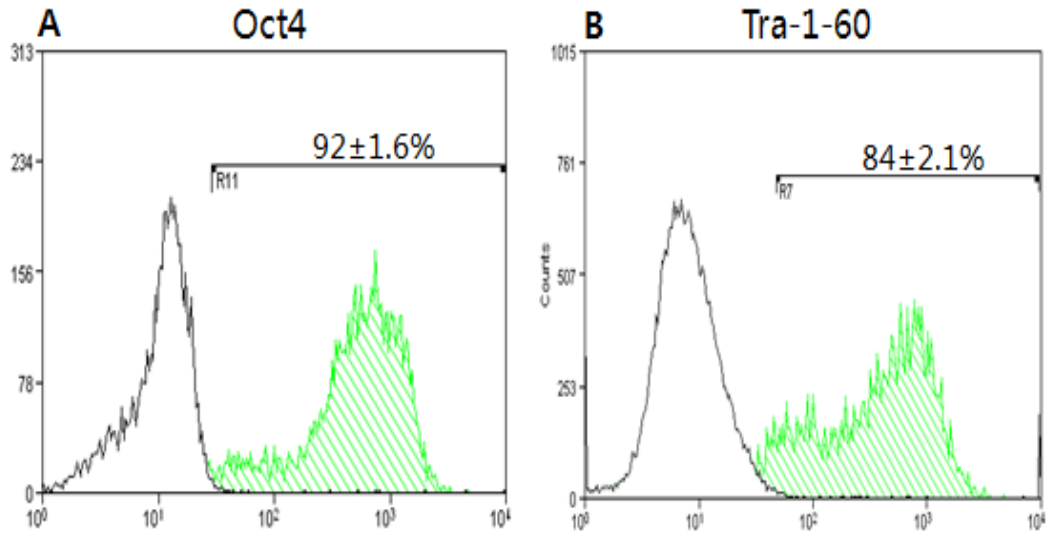


Figure 3.3 Flow cytometry analysis of Oct4 and Tra-1-60 expression level. (A and B) Flow cytometry analysis revealed that iPSCs expressed a high level of Oct4 and Tra-1-60 respectively. Each histogram represents a negative population stained with the corresponding isotype and a secondary antibody (blank) and samples stained with a primary and a secondary antibody (dashed).

3.2.2. Pluripotency of MSUH001-iPSCs

To examine whether iPSCs spontaneously differentiate into three germ layers, iPSCs colonies were removed from the feeder layer and cultured in suspension without bFGF for 10 days. They began to form a spheroid structure termed EBs on D2 of suspension culture and they were fixed on D10 to analyze expression of the germ layer markers. Immunocytochemistry revealed that EBs contained cells positive for Nestin (ectoderm lineage), Brachyury (mesoderm lineage) and Sox17 (endoderm lineage) without any preferred trajectories (Fig 3.4).

Figure 3.4

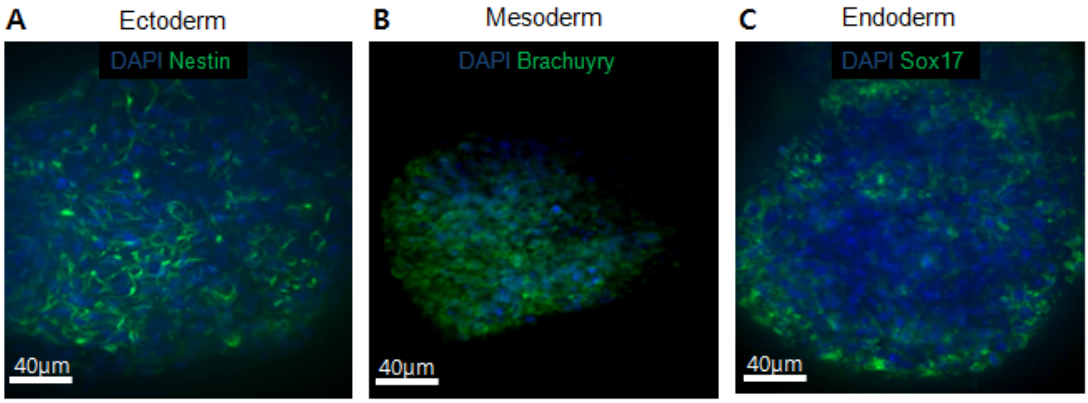


Figure 3.4 Three germ layer differentiation of MSUH001-iPSCs. iPSCs colonies were detached from the feeder layer and cultured for 10 days in suspension as EBs in DMEM supplemented with 10% KOSR. The EBs differentiated into (A) ectoderm, (B) mesoderm and (C) endoderm.

3.2.3. Karyotype analysis

In order to ensure the safety of stem cell based therapy, it is essential to check whether cells maintain the correct number and structure of chromosomes. Pluripotent stem cells are prone to chromosomal changes and transplantation of differentiated cells from pluripotent stem cells carrying abnormal chromosomes leads to the formation of a cancer-like structure (Moon et al., 2011(a)). Our karyotype analysis revealed that iPSCs were karyotypically normal (Fig 3.5).

Figure 3.5

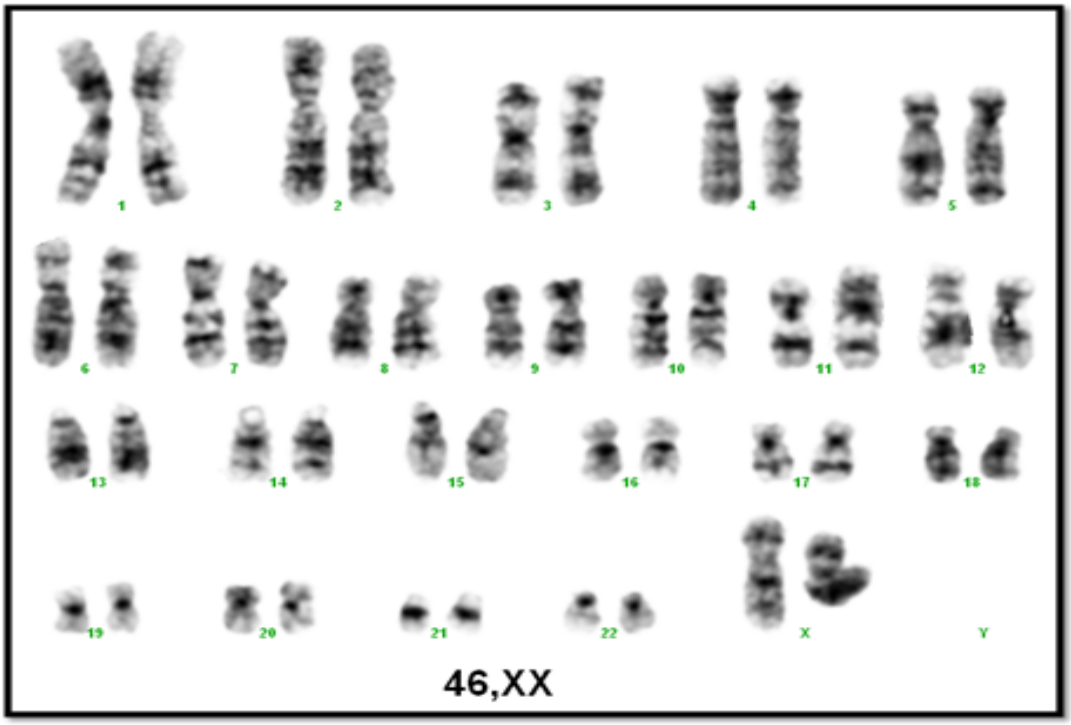


Figure 3.5 Karyotype analysis of MSUH001-iPSCs (P34). The cells were karyotypically normal with the correction number of chromosomes.

3.3. Characterization of Shef3-hESCs

3.3.1. Morphology and undifferentiated state

hESCs were first established in 1998 by isolating the inner cell mass from the human blastocyst (Thomson et al., 1998). In line with iPSCs culture system, hESCs are typically cultured in the presence of MEF feeder layers to support undifferentiated state in the culture. However, this culture condition is viewed as inappropriate for clinical purposes due to cross contamination between the two species (Mallon et al., 2006). For this reason, hESCs have also been cultured with human embryonic fibroblasts (HEFs) or in feeder-free culture system using commercially available extracellular matrices (Amit et al., 2003; Hernandez et al., 2010). hESCs have long been thought as an ideal source materials for cell replacement therapy and over a decade of research have led to the first clinical trial of hESCs based therapy (see table 1.2). In this experiment, we used Shef3-hESCs which were co-cultured with Mytomycin-C inactivated MEF feeder cells. Two days after transferring to new feeder cells, they formed colonies that exhibited typical hESCs morphology of flat and round colonies tightly packed with single hESCs (Fig 3.6A) (Reubinoff et al., 2000). All the colonies in the culture expressed pluripotency and undifferentiated markers Oct4 and Tra-1-60 detected by immunocytochemistry (Fig 3.6C) and flow cytometry revealed that cultures contained a high percentage of Oct4 and Tra-1-60 expressing cells (Fig 3.7A and B).

Figure 3.6

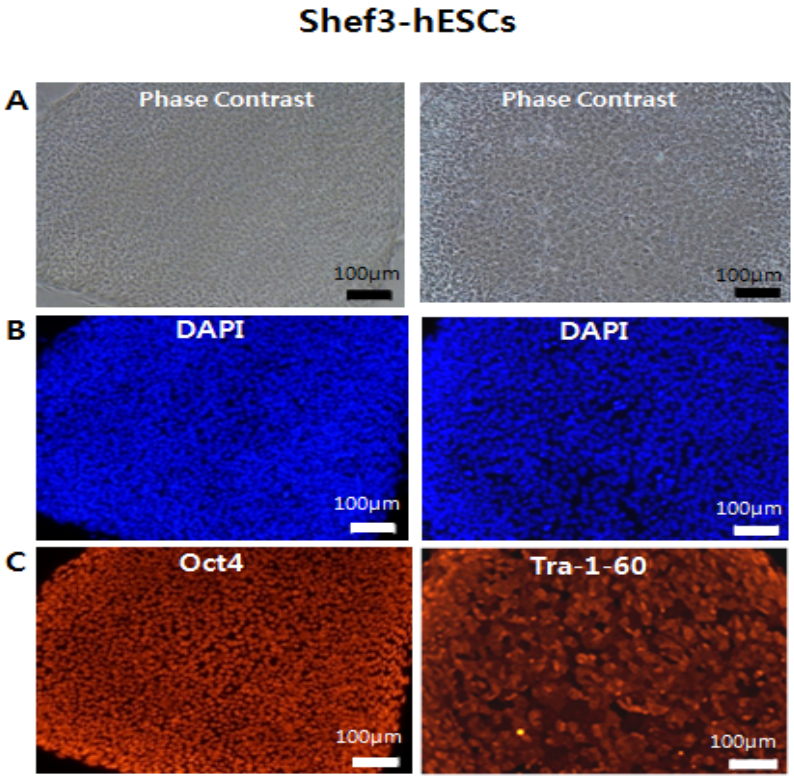


Figure 3.6 Characterisation of Shef3-hESCs. (A) hESCs maintained on MEF feeder layer formed round, tightly packed, compact colonies (B and C) Immunocytochemical analysis showed that hESCs expressed undifferentiated markers Oct4 and Tra-1-60 in the nucleus and cytoplasm respectively. (D and E) Flow Cytometry analysis of the undifferentiated markers (Oct4 and Tra-1-60) expression level hESCs expressed high level of each marker. Each histogram represents a negative population stained with the corresponding isotype and a secondary antibody (blank) and samples stained with a primary and a secondary antibody (dashed).

Figure 3.7

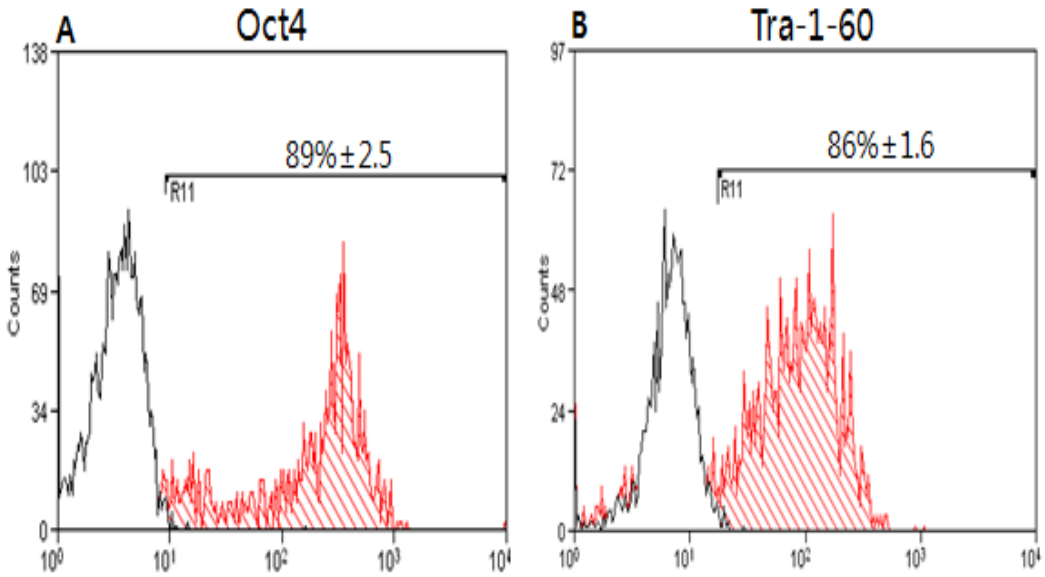


Figure 3.7 Flow cytometry analysis of Oct4 and Tra-1-60 expression level. (A and B) Flow Cytometry analysis of the undifferentiated markers (Oct4 and Tra-1-60) revealed that hESCs expressed high level of each marker. Each histogram represents a negative population stained with the corresponding isotype and a secondary antibody (blank) and samples stained with a primary and a secondary antibody (dashed).

3.3.2. Pluripotency of Shef3-hESCs

Shef3-hESCs were allowed to spontaneously differentiate into cells representing all three embryonic germ layers through EB formation. The colonies were removed from the feeder layer and cultured in suspension in the same condition as iPSCs-EBs. They began to form spheroid aggregates of cells D2 after suspension culture and were allowed to grow until D10 when immunocytochemistry was performed to analyse the expression of three germ layer markers. The analysis revealed that Nestin-positive cells, representing ectodermal lineage, were localized throughout the surface of EBs (Fig 3.6.). Brachyury- and Sox17- positive cells, representing mesoderm and endoderm respectively, were also generated after culturing EBs for 10 days (Fig 3.8).

Figure 3.8

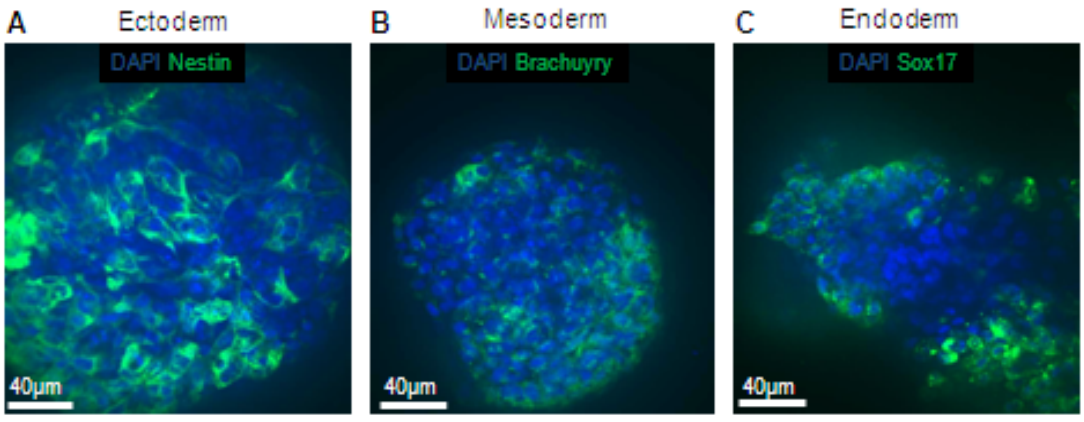


Figure 3.8 Three germ layer differentiation of Shef3-hESCs. hESCs colonies were detached from the feeder layer and cultured for 10 days in suspension as EBs in DMEM supplemented with 10% KOSR. The EBs differentiated into (A) ectoderm, (B) mesoderm and (C) endoderm.

3.3.3. Karyotype analysis

Karyotype analysis of Shef3-hESCs revealed that cells were karyotypically normal, maintaining the correct number and structure of chromosomes during the culture (Fig 3.9).

Figure 3.9

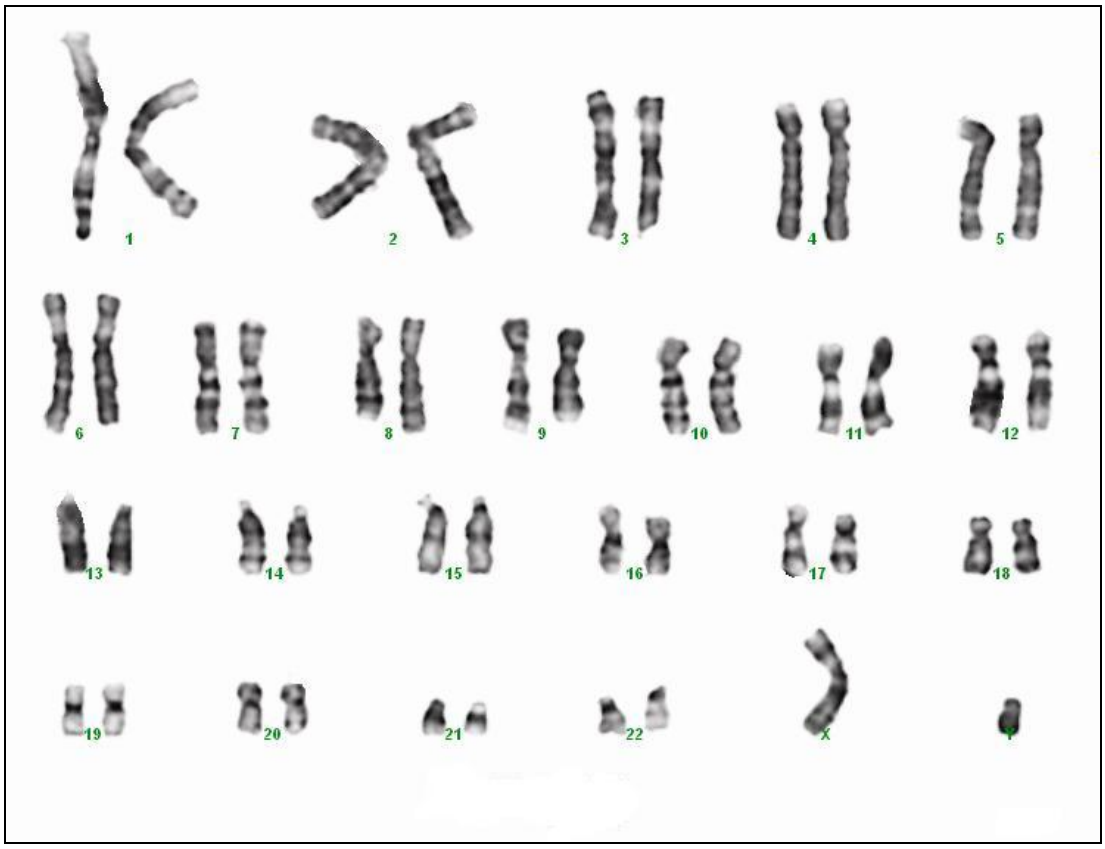


Figure 3.9 Karyotype analysis of Shef3-hESCs (P55). The cells were karyotypically normal with the correction number of chromosomes.

3.4. Discussion

Under the appropriate culture regimes, human iPSCs and hESCs have the property to differentiate into any types of cells in the body thus, making them an ideal source material for cell replacement therapy. Prior to any differentiation studies, it is essential to evaluate cells maintain this potential and are uncommitted to any lineages differentiation until they receive differentiation stimuli. In this chapter, we demonstrated that two human pluripotent stem cell lines remained undifferentiated in the culture whilst maintaining the ability to differentiate into all three embryonic germ layers. MSUH-iPSCs and Shef3-hESCs lines were chosen because the cell lines have been well characterized, exhibiting all the aspects of true human pluripotent stem cell characteristics (MSUH001-iPSCs by the Cibelli's group, Michigan State University and the Spanish Stem Cell Bank, and Shef3-hESCs by the Andrew's group, Sheffield University and the UK Stem Cell Bank). For this reason, it was decided that these cell lines provide a good candidate for examining the effect of lowering O_2 tension during retinal differentiation (see chapter 4 and 5).

This evaluation was particularly important for characterization of MSUH001-iPSCs line. Shef3-hESCs culture was seemingly homogenous in appearance, exhibiting typical morphology of hESCs colonies (Fig 3.6A). In addition, immunocytochemistry and flow cytometry showed that the culture expressed a high percentage of Oct and Tra-1-80 (Fig 3.7A and B) and differentiated into cells

representing all three embryonic germ layers (Fig 3.8).

MSUH001-iPSCs culture however, contained a mixture of morphologically varable hESCs-like colonies which led us to investigate the identity of different looking colonies. Immunocytochemistry against Oct4 revealed that the culture contained a mixture of colonies that did not express (type I), partially expressed (type II) and fully expressed Oct4 (type III) (Fig 3.1B). The heterogeneicy nature of human pluripotent stem cell culture could severely hinder their ability to generate specific types of cells. It was revealed that fibroblasts that are partially reprogrammed to exhibit hESCs-like morphology failed to form teratoma or differentiate into all three embryonic germ layers (Chan et al., 2009). In order to obtain a homogenous population of fully reprogrammed cell colonies, we selectively picked colonies that exhibited type III colony morphology and expanded further (Fig 3.1B bottom row). After 7 passages, our immunocytochemistry and flow cytometry revealed a comparable expression level of Oct4 and Tra-1-80 to Shef3-hESCs (Fig 3.3A and B). In addition, when colonies were removed from the feeder layer and cultured in suspension in the absences of bFGF, they differentiated cell representing all three embryonic germ layers, confirming the pluripotent nature of the cells (Fig 3.4).

It has been revealed that reprogramming efficiency is very low and cultures are thought to be heterogeneous, containing mixed population of none, partially and

fully reprogrammed cells despite acquiring hES-like morphology (Chan et al., 2009). It has been believed that reprogramming techniques are still to be optimized (Feng et al., 2009). In addition, one study revealed that not all fibroblasts are susceptible to reprogramming. The researchers revealed that only a small population of cells termed multilineage-differentiating stress-enduring (Muse) cells within human fibroblast population exclusively reprogrammed to become true iPSCs (Wakao et al., 2011). Efforts have been made to enhance reprogramming efficiency using various methods that include novel gene delivery systems and the use of low O_2 during reprogramming processes (see section 1.2.2.3 and Yoshida et al., 2009).

3.5 Conclusion

In this chapter, it was confirmed that the two human pluripotent stem cell lines MSUH001-iPSCs and Shef3-hESCs exhibit the true characteristics of pluripotent stem cells and could be used as a starting material for the generation of retinal cells which are described in detail in the next chapter.

4. Generation of RPCs from MSUH001-iPSCs and Shef3-hESCs

4.1. Introduction

Human pluripotent stem cells hold the promise for developing a novel treatment for currently incurable degenerative medical conditions (Oliveri and Anderson, 2006). One of the conditions that can be benefited from stem cell based therapy is genetic ocular diseases such as retinitis pigmentosa (RP) that is characterized by the loss of photoreceptors in the retina (see section 1.1.2). Using combinations of forebrain inducing growth factors such as DKK-1 (wnt pathway antagonist), Noggin (BMP pathway antagonist) and Lefty A (nodal pathway antagonist), it has been possible to generate RPCs from pluripotent stem cells with varying efficiencies (Lamba et al., 2006; Osakada et al., 2008; Meyer et al., 2010). Generating RPCs from human pluripotent stem cells has opened a new paradigm for the treatment of photoreceptor dystrophic conditions. In particular, Lamba et al. generated ~80% RPCs co-expressing RPC markers Pax6 and Chx10 21 days after plating hESCs-EBs (Lamba et al., 2006). Further studies revealed that transplanting photoreceptor precursor cells derived from RPCs significantly improved vision in photoreceptor-deficiency mice (Lamba et al., 2010). Despite the promising evidence, it is a widely accepted that differentiation efficiency varies greatly between different

pluripotent stem cell lines (Osakada et al., 2008), thus may result in lower yield of target cells. The Lamba protocol was chosen because when this study was conducted, only two retinal differentiation protocols were available (see section 1.3). The retinal differentiation by Takahashi group was lengthy involving complicated steps whereas the Lamba protocol seemed more reproducible, thus the latter protocol was chosen.

4.1.1. Aim

The aim of this chapter is to evaluate whether MSUH001-iPSCs and Shef3-hESCs are suitable cells lines for generating RPCs with a comparable efficiency using the Lamba protocol. This chapter provides the control data against which any benefits of operation under hypoxic conditions can be quantified in the following chapter.

4.2. Generation of RPCs from MSUH001-iPSCs

4.2.1. EB formation and induction of early eye field gene

iPSCs colonies were removed from the feeder layer and cultured in suspension in the medium supplemented with DKK-1, Noggin and IGF-1 for 3 days. The floating colonies of cells formed aggregates of cells after 1 day being in suspension culture that further developed into a spheroid structure on day 3 (Fig 4.1A). To examine whether the supplement of the growth factor favoured the induction of early eye field genes, we analysed the expression level of early eye field genes Six3 and Lhx2. These are early eye field genes required for retinal specification and optic cup development respectively and has been reported that Lhx2 defines presumptive retina field and transactivates Six3 expression in the same domain (Tetreault et al., 2009; Liu et al., 2010). The qPCR analysis revealed that supplement of the growth factors significantly increased the expression level of Six3 by a 2.2 fold compared to EBs differentiating without the growth factors (Fig 4.1B, $p \leq 0.05$). Although the expression level of Lhx2 in EBs differentiating in the presence of the growth factor increased compared to EB differentiating without the growth factors, the increase deemed to be statistically not significant (Fig 4.1B).

Figure 4.1

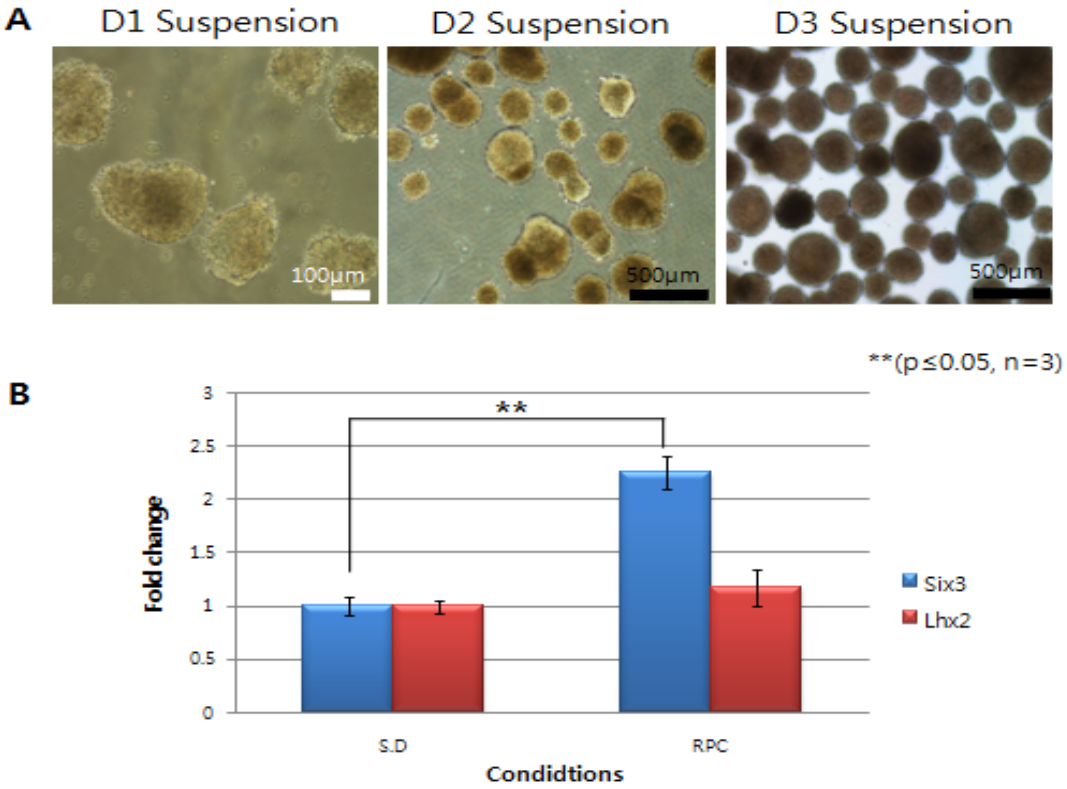


Figure 4.1 iPSCs-EB formation and activation of the early eye field genes. A) iPSCs-EB formation in the presence of DKK-1, Noggin and IGF-1 was observed for three days. B) The expression level of the early eye field genes Six3 and Lhx2 in iPSCs-EBs formed in the presence of the growth factors (RPC) was compared to spontaneously differentiating iPSCs-EBs (S.D). The analysis revealed a significant increase in Six3 expression in iPSCs-EBs formed with growth factors compared to spontaneously differentiating iPSCs-EBs (p p≤0.05, n=3). However, a small increase was observed for Lhx2 which deemed to be insignificant compared to spontaneously differentiating iPSCs-EBs.

4.2.2. Adherent retinal differentiation of iPSCs-EBs

After culturing EBs for 3 days in suspension, EBs were transferred to Matrigel-coated wells for the development of retinal cells. For this adherent culture, KOSR was withdrawn and N2 supplement and bFGF were additionally supplemented which have been thought to promote photoreceptor differentiation (Hicks and Courtois, 1992). Three days after plating EBs, cells migrated as tightly packed neuroepithelial cells from the site at which the EBs had attached in a similar pattern as previously observed (Fig 4.2A, Meyer et al., 2009). Some cells expressed a neural stem cell marker Nestin, suggesting that they had begun to differentiate into neuroectodermal lineage (Fig 4.2A bottom). At this time point, some cells in the EBs still expressed pluripotency marker Oct4 (Fig 4.2A middle). 6 days after plating EBs, it was observed that EBs continued to differentiate into Nestin-positive cells but some populations of cells were still positive for Oct4 (Fig 4.2B). 10 days after plating EBs, densely-packed populations of cells arranged in a radial manner, termed as neural rosettes due to their resemblance to early neural tube development (Zhang et al., 2001) formed in close proximity to the original site of attachment (Fig 4.2C asterisks). Cells around the rosettes were still positive for Oct4 and Nestin (Fig 4.2C middle and bottom). 17 days after plating EBs, it was revealed that cells no longer showed positive staining for both Nestin and Oct4 (Fig 4.2D). Cells did not show positive staining for RPC markers Pax6 and Chx10 at all the time points data not

shown

Figure 4.2

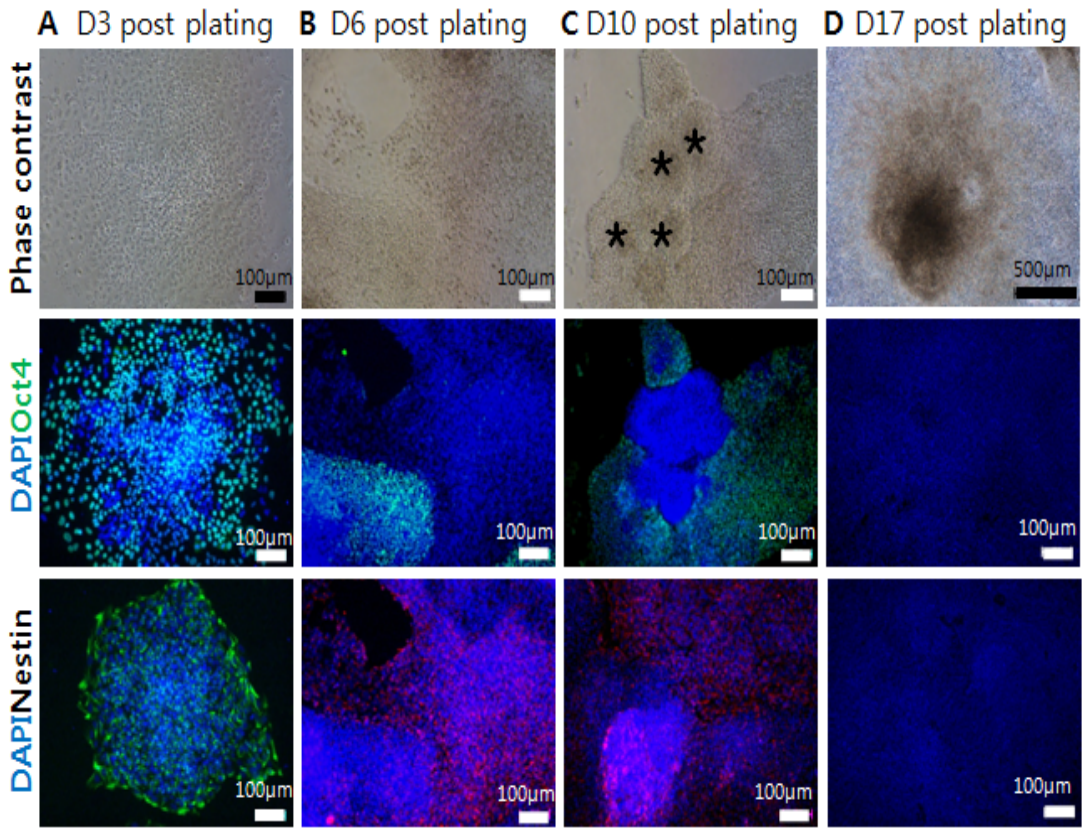


Figure 4.2 Expression of pluripotency (Oct4) and neural stem cell marker (Nestin) during the adherent differentiation. 3 days post plating iPSCs-EBs, many cells still expressed Oct4 where EBs were attached. At the same time point, it was also evident that EBs had already differentiated into Nestin positive neural stem cells. 6 days post plating, cells developing from the attached EBs were predominantly Nestin positive neural stem cells and although Oct4-positive cells were still present. 10 days post plating saw the formation of neural rosettes (C asterisks) that were both Oct4- and Nestin-negative but surrounding cells still expressed the proteins. Both proteins were undetectable from 17 days post plating.

4.2.3. Generation of Pax6 and Chx10 co-expressing RPCs from iPSCs

Pax6 and Chx10 are key transcription factors required for RPC multipotency and proliferation (Liu et al., 1994; Marquardt et al., 2001) and it has been shown that cells expressing these can be generated from human pluripotent stem cells 21 days after plating EBs in the presence of DKK-1, Noggin and IGF-1 (Lamba et al., 2006). Using immunocytochemistry, we confirmed the generation of RPCs co-expressing Pax6 and Chx10 from MSUH001-iPSCs. The areas of Pax6 positive cells were abundant throughout the culture but only a few Pax6-positive colonies co-expressed Chx10 (Fig 4.3). Quantification of iPSCs-RPCs using flow cytometry revealed that 74±4.6% were Pax6 positive, 31±2.8% expressed chx10 and 31±2.1% co-expressed Pax6 and Chx10 21 days after plating EBs (Fig 4.4A and B). The expression of rod precursor marker Nrl and mature photoreceptor markers S-Opsin and Rhodopsin were also investigated by immunocytochemistry but they were undetectable, suggesting that the differentiation culture period was not sufficiently long enough (data now shown). At the same time point, we performed qPCR to examine whether retinal genes were induced from pluripotent iPSCs. It was observed that there was a significant 3.7- and 11.2- fold increase in the expression level of Pax6 and Chx10 respectively compared to pluripotent iPSCs ($p \leq 0.05$ Fig 4.5). Rx, another member of early eye field genes known to play roles in the retinal development, showed a significant increase compared to pluripotent iPSCs (5.9 fold increase, $p \leq 0.05$ Fig 4.5).

However, at this time point there were no significant increases in the expression levels of early eye field markers such as Lhx2, Otx2 and Six3 compared to pluripotent iPSCs ($p>0.05$ in all cases).

Figure 4.3

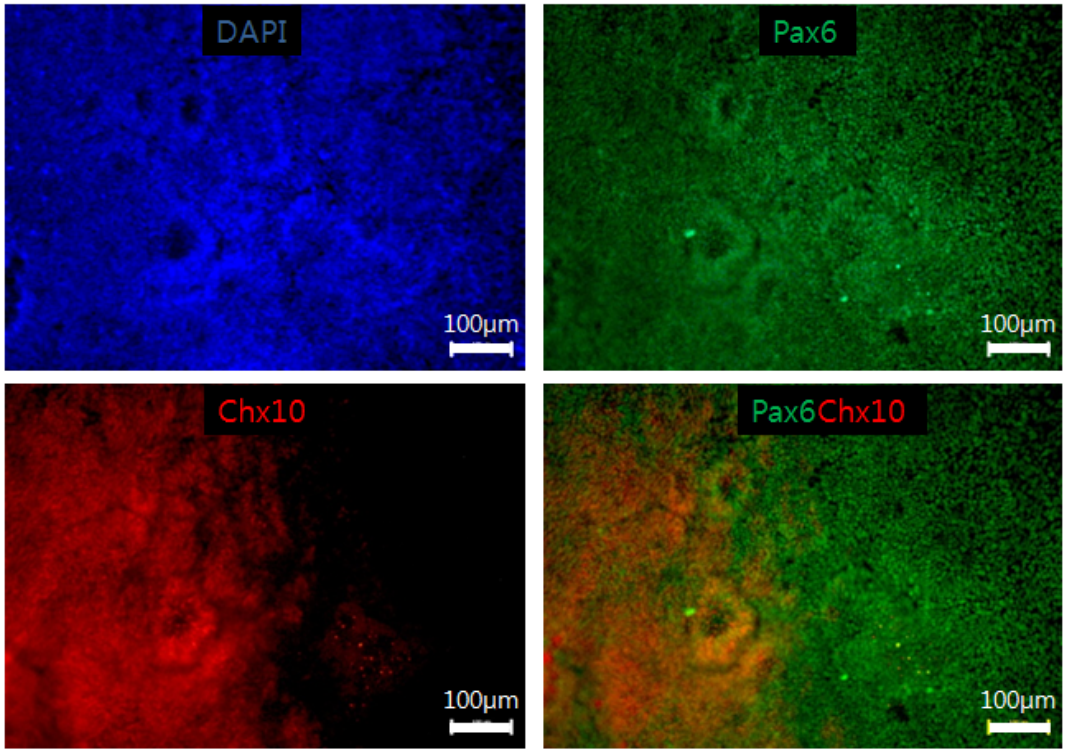


Figure 4.3 Analysis of Pax6 and Chx10 co-expressing RPCs 21 days post plating EBs using immunocytochemistry. Representative images of colonies co-expressing Pax6 and Chx10

Figure 4.4

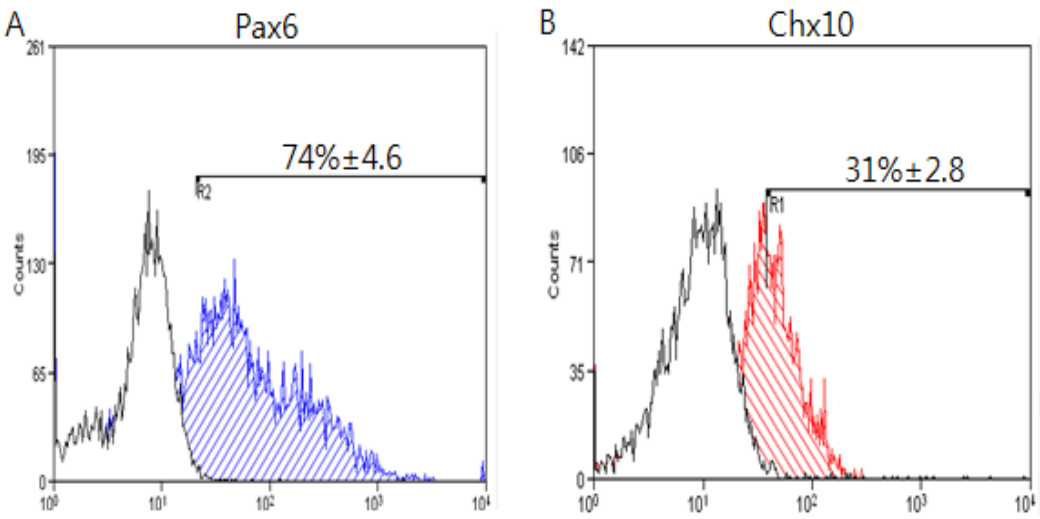


Figure 4.4. Flow cytometry analysis of Pax6 and Chx10 expression level. (A and B) The percentage of Pax6 and Chx10 expressing cells 21 days after plating EBs. Each histogram represents a population stained with the corresponding isotype and secondary antibody (blank) and with primary and secondary (dashed). The standard error is shown as a result of three independent experiments (n=3).

Figure 4.5

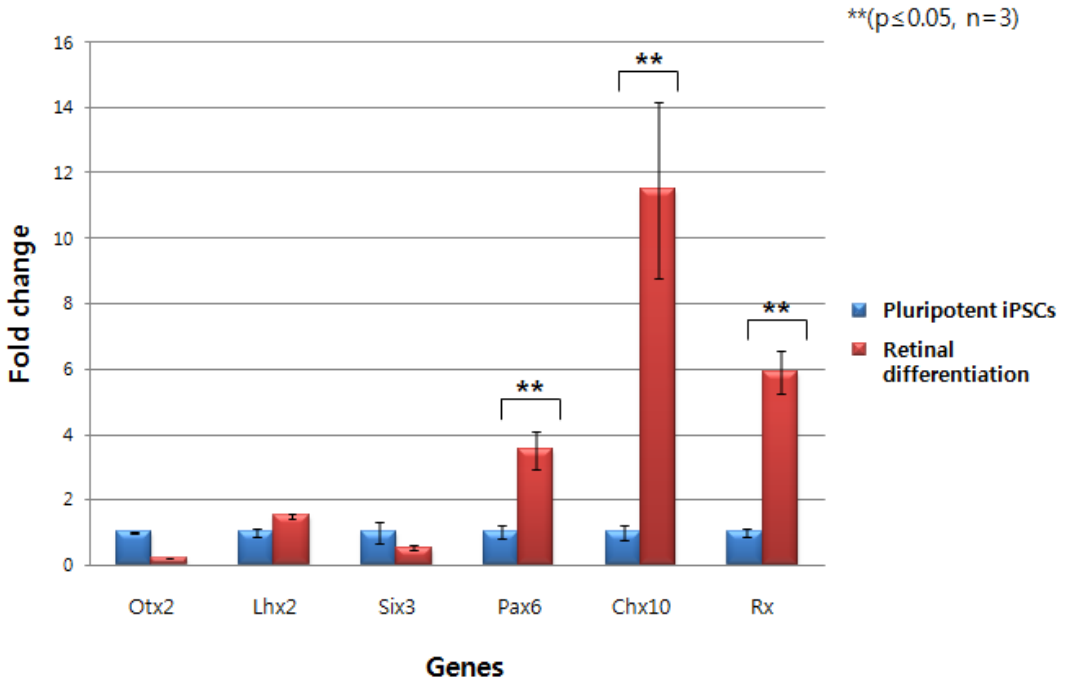


Figure 4.5. Relative mRNA expression of the retinal markers 21 days after plating iPSCs-EBs. The expression level of RPC markers Pax6 and Chx10 significantly increased compared to pluripotent iPSCs 21 days post plating EBs. Out of the early eye field markers, only Rx showed a significant increase in the expression level compared to pluripotent iPSCs. The blue and red bars represent pluripotent iPSCs and retinal differentiation of iPSCs respectively.

4.3. Generation of RPCs from Shef3-hESCs

4.3.1. EB formation and induction of early eye field genes

Shef3-hESCs were removed from the MEF feeder layer and formed EBs in the presence of the same growth factors. They formed EBs which were indistinguishable in terms of size and morphology from iPSCs-EBs after 3 days in suspension culture (Fig 4.6A). Analysis of the expression level of Six3 and Lhx2 by qPCR revealed a significant 2.3 fold increase in the expression level of Six3 in EBs differentiating with the growth factors compared to spontaneously differentiating EBs ($p \leq 0.05$ Fig 4.6B). This pattern is consistent with iPSCs-EBs differentiation in the presence of the growth factors. Unexpectedly, the expression level of Lhx2 decreased in EBs differentiating with the growth factors compared to spontaneously differentiating EBs though this decrease was statistically not significant (Fig 4.6B).

Figure 4.6

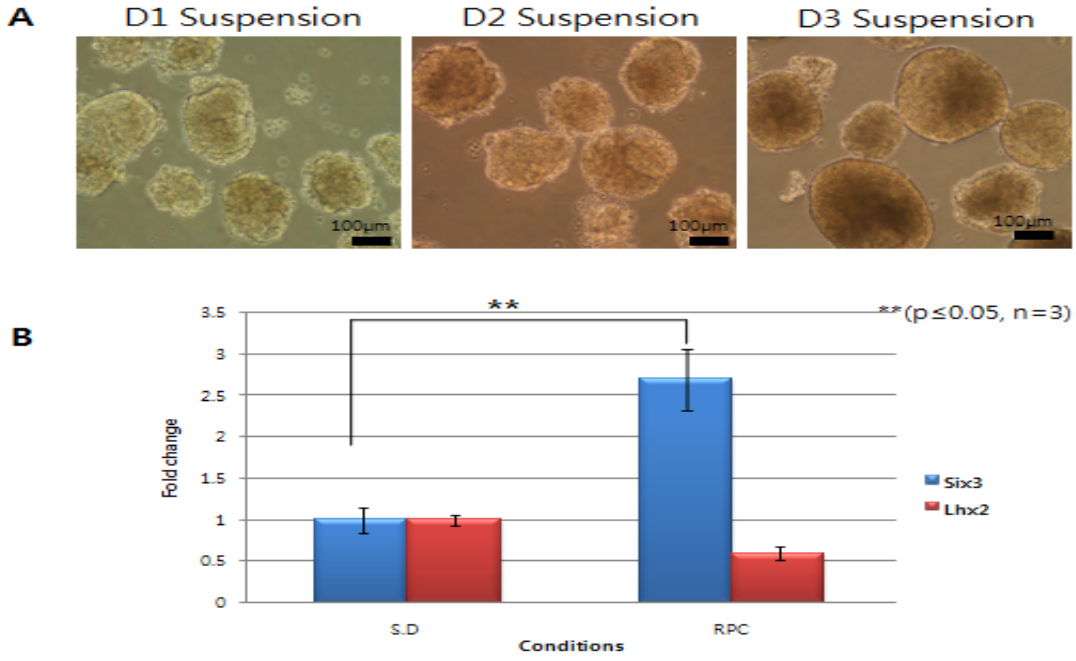


Figure 4.6 hESCs-EB formation and activation of the early eye field genes. A) hESCs-EB formation in the presence of DKK-1, Noggin and IGF-1 was observed for three days. B) The expression level of the early eye field genes Six3 and Lhx2 in hESCs-EBs formed in the presence of the growth factors (RPC) was compared to spontaneously differentiating hESCs-EBs (S.D). The analysis revealed a significant increase in Six3 expression in hESCs-EBs formed with growth factors compared to spontaneously differentiating hESCs-EBs (p $p \leq 0.05$, n=3). However, Lhx2 expression level decreased in hESCs-EBs formed in the presence of the growth factors compared to spontaneously differentiating hESCs-EBs although the decrease was statistically not significant. The blue and red bar represents Six3 and Lhx2 expression level respectively.

4.3.2. Adherent retinal differentiation of Shef3-EBs

3 days after plating hESCs-EBs, there appeared to be a few neurite processes on the peripheral region of the attached EBs (Fig 4.7A arrow). Immunocytochemistry at this time point revealed that EBs contained a small population of Oct4-positive cells and the majority of cells expressed Nestin (Fig 4.7A middle and bottom). The same observation was made 6 days after plating EBs as the culture contained a mixture of cells stained positive for Oct4 and Nestin, although Oct4-positive cells were sparsely populated (Fig 4.5B). Twelve days after plating EBs, neural rosettes were formed (Fig 4.7 asterisks) and the majority of cells expressed Nestin and only very small populations of cell expressed Oct4 (Fig 4.7C). The expression of Oct4 and Nestin was completely down-regulated 18 days after plating EBs and the morphology became increasingly difficult to define using phase contrast microscopy as cells became multilayered (Fig 4.7D). In consistent with differentiation of iPSCs-EBs, cultures did not show positive staining for Pax6 and Chx10 at all the time points (date not shown).

Figure 4.7

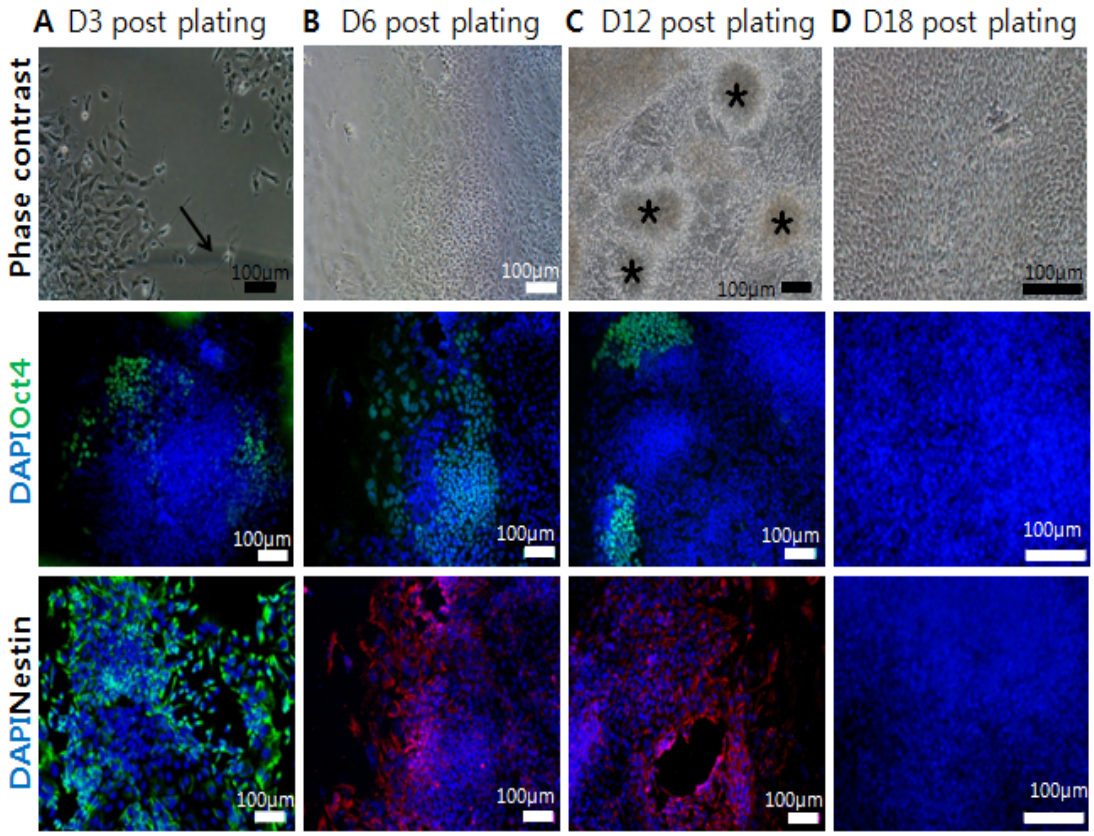


Figure 4.7 Expression of Oct4 and Nestin during adherent differentiation of hESCs-EBs. 3 days posting hESCs-EBs, small population of Oct4 positive cells were observed and the majority of cells developing from the plated hESCs-EBs were Nestin-positive cells. These markers were still present 8 days post plating EBs and 12 days post plating hESCs-EBs saw the formation of neural rosettes. By this time point, Oct4-positive cells were very sparse and many cell expressed Nestin. Both markers were undetectable 18 post plating.

4.3.3. Generation of Pax6 and Chx10 co-expressing RPCs from hESCs

Performing immunocytochemistry 21 days after plating EBs revealed that, in consistent with iPSCs differentiation, a large population of cells expressed Pax6 (Fig 4.8A). However, it was visually confirmed that cells co-expressing Pax6 and Chx10 were rare within in the culture (Fig 4.8A-C). Indeed, quantification using flow cytometry revealed that only 1% of the cultures co-expressed Pax6 and Chx10 (Fig 4.9A and B). To find out whether cells with retinal genotype had produced from pluripotent hESCs, we analyzed the expression level of early eye field and RPC genes 21 days after plating EBs. The analysis revealed a significant 9.9- and 9.4- fold increase in the expression level of Pax6 and Chx10 respectively when compared to pluripotent hESCs (Fig 4.10). In consistent with iPSCs differentiation, the expression level of Rx also increased 21 days after plating EBs but this increase were found to be statistically not significant. Surprisingly, Lhx2 which is expressed during the early stage of retinal development, was up-regulated in the differentiating cells compared to pluripotent hESCs 21 days after plating EBs (8.7-fold increase, $p \leq 0.05$, Fig 4.10). Retinal genes associated with early eye field specification (Otx2 and Six3) were significantly down-regulated at the end of D21 differentiation, suggesting that retinal differentiation had surpassed early retinal specification stage ($p \leq 0.01$, Fig 4.10)

Figure 4.8

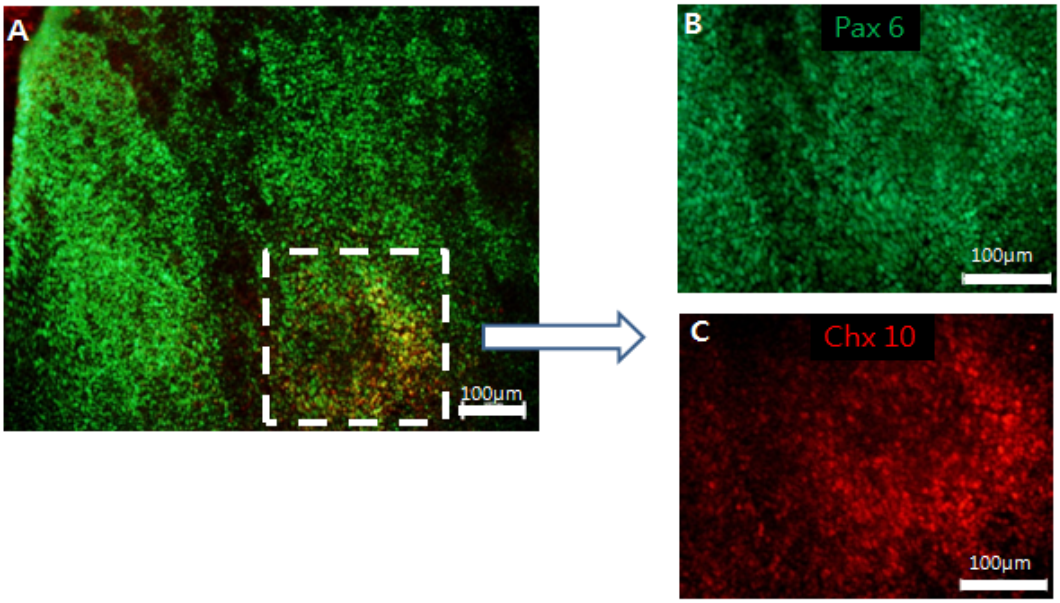


Figure 4.8 Analysis of Pax6 and Chx10 co-expressing RPCs 21 days post plating EBs. (A) Representative images of colonies co-expressing Pax6 and Chx10. (B and C) enlarged image of Pax6 and Chx10 co-expressing cells.

Figure 4.9

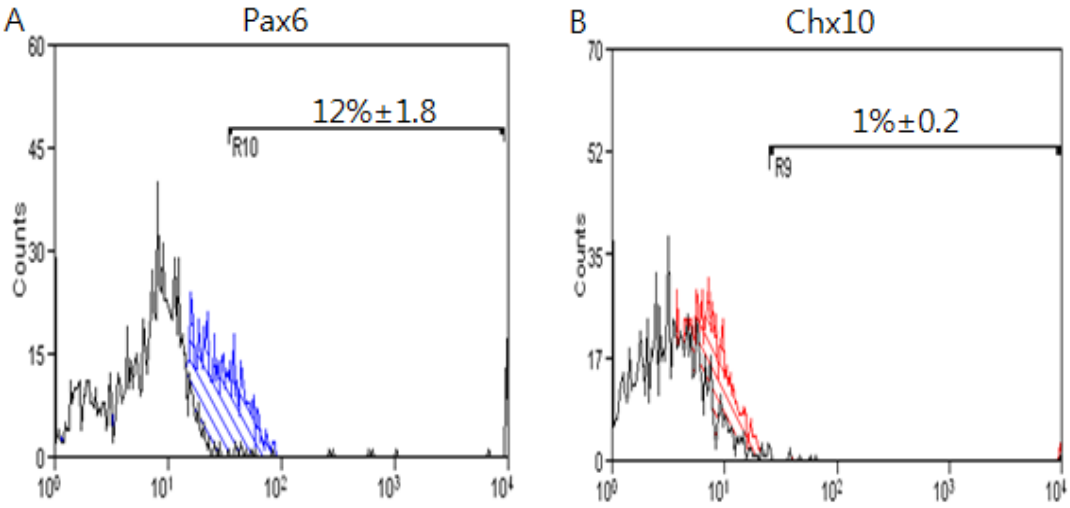


Figure 4.9. Flow cytometry analysis of Pax6 and Chx10 expression level. (A and B) The percentage of Pax6 and Chx10 expressing cells. Each histogram represents a population stained with the corresponding isotype and secondary antibody (blank) and with primary and secondary (dashed). The standard error is shown as a result of three independent experiments (n=3).

Figure 4.10

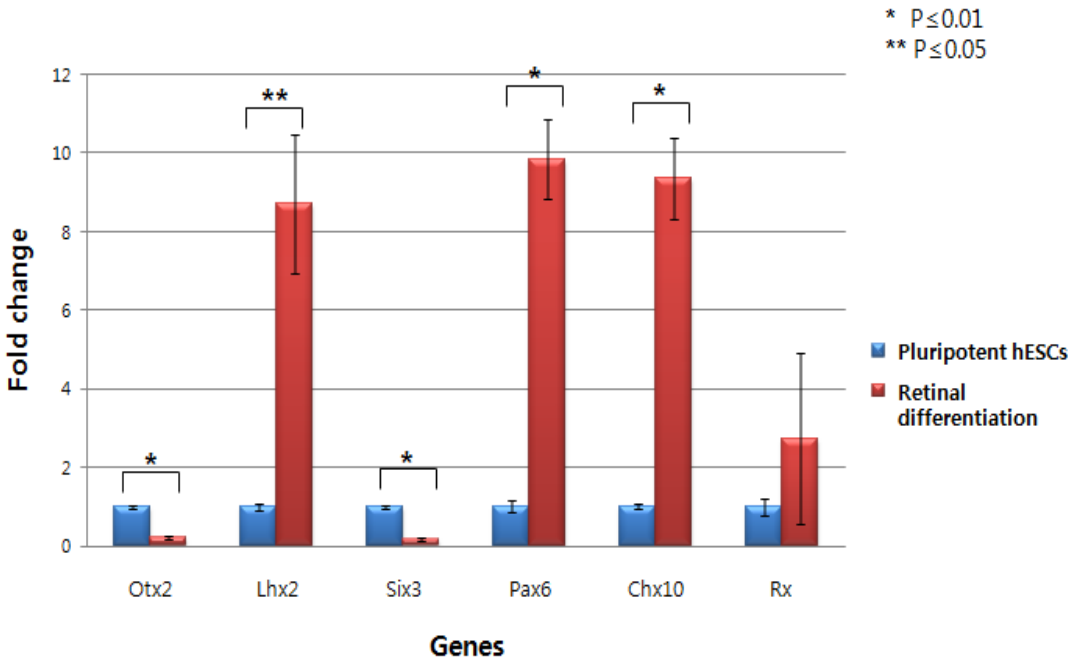


Figure 4.10. Relative mRNA expression of the retinal markers 21 days after plating hESCs-EBs. The expression level of RPC markers Pax6 and Chx10 significantly increased compared to pluripotent hESCs 21 days post plating EBs. Out of the early eye field markers, only Lhx2 showed a significant increase in the expression level compared to pluripotent hESCs. The blue and red bar represents pluripotent and retinal differentiation of Shef3-hESCs respectively.

4.4. Discussion

Since the establishment of human pluripotent stem cells, many protocols have been developed to generate specific cell types for potential therapeutic application. However, it is often found that differentiation protocols are cell line dependent as differentiation efficiency varies greatly across the different human pluripotent stem cell lines. In 2006, Lamba et al. developed the first differentiation protocol for generating RPCs from H1 hESCs line (the first hESCs line created by Thomson's group in 1998). The protocol involves the application of various growth factors such as DKK-1, Noggin and IGF-1 that are reported to induce forebrain development during early mammalian development (Lamba et al., 2006). The results demonstrated the generation of ~80% RPCs co-expressing RPC markers Pax6 and Chx10 and this protocol was validated using three different iPSCs lines with a comparable efficiency. In this chapter, it was first examined in the lab, whether the Lamba differentiation protocol can be applied to generate RPCs from Shef3-hESCs and MSUH001-iPSCs lines with a comparable efficiency. In this chapter, it was confirmed 1) that both cell lines failed to achieve a comparable RPC differentiation efficiency as H1-hESCs line, 2) differences in the morphological characteristics during retinal differentiation and 3) that MSUH001-iPSCs line differentiated into RPCs better than Shef3-hESCs.

The protocol consists of two stages; the first stage involves culturing cells as

floating aggregates termed EBs for first three days and the second stage involves an adherent culture of EBs for 21 days after the EB stage. iPSCs- and hESCs-EBs formed in the presence of the growth factors were morphologically indistinguishable (Fig 4.1A and 4.6A). The expression level of Six3, a gene expressed during early eye field specification, significantly increased compared to spontaneously differentiating EBs without the growth factors (Fig 4.1B and 4.6B). Lhx2, another gene reportedly required for the early eye field specification did not show any significant changes in the expression level in EBs differentiated with growth factors compared to spontaneously differentiating EBs (Fig 4.1B and 4.6B). This was the phenomenon observed in both MSUH001-iPSCs and Shef3-hESCs lines, suggesting that the protocol is effective in inducing Six3 expression 3 days after culturing EBs (Fig 4.1B and 4.6B).

Both cell lines were indistinguishable genotypically and phenotypically during the first stage of the protocol but differences were observed between the cell lines during the adherent culture of EBs. We observed morphological differences when both pluripotent cell types were induced to differentiate into Pax6 and Chx10 co-expressing RPCs. When hESCs-EBs were plated, spindle cells with neurite processes developed on the peripheral region of the attached EBs (Fig 4.7 arrow). However, the development of this neuronal morphology was not observed when

iPSCs-EBs were attached. Instead, cells migrated outwards from the attached site as tightly packed neuroepithelial colonies similar to those observed in a previous study (Fig 4.2A). The expression profile of Oct4 and Nestin was similar between the two cell lines during the differentiation. However, it was noticeable that the population of Oct4-positive cells was visually more abundantly sustained throughout the differentiation period up to 15 days after plating iPSCs-EBs compared to hESCs-EBs (Fig 4.2 and 4.7).

21 days post plating EBs, it was revealed the generation of RPCs co-expressing Pax6 and Chx10 through performing immunocytochemistry. To quantify the percentage of RPCs generated, flow cytometry was performed to reveal retinal differentiation of Shef3-hESCs was inefficient, generating just over 1% of RPCs co-expressing Pax6 and Chx10 (Fig 4.6D). In contrast, iPSCs generated more cells expressing Pax6 and Chx10 (31±2.1%) at the same condition (Fig 4.3B).

Reasons for variations in differentiation efficiency across the different cell lines are subject to much debate. It has been reported that during embryogenesis, the ICM is heterogeneous as early as embryonic day 3.5 (E3.5) when subsets of cells begin to differentiate into primitive endoderm and epiblast (Chazaud, 2006). This early differentiation commitment of the ICM has been confirmed in a recent study which showed a differential expression of GATA6 (primitive endoderm marker) and

Nanog (pluripotency marker) in mESCs (Singh, 2007). As the primary culture of hESCs involves isolation of clumps of cells from this heterogenous ICM as opposed to from a single parental cell, *in vitro* ESC cultures always contain populations of ESCs with different developmental potentials (Shamblott, 1998; Thomson., 1998; Reubinoff., 2000). An earlier study by Osakada et al. (Osakada et al., 2008) revealed significant variation in the efficiency between two hESCs lines during the retinal differentiation. This may indicate that heterogeneous ESCs contain a subpopulation of cells which are refractory to retinal differentiation. We have hypothesized that differences between MSUH001-iPSCs and Shef3-hESCs may be due to amount of refractory cells present in each cell type. In addition it is possible that the reprogramming process may enhance the retinal differentiation of the pluripotent cells derived.

4.5 Conclusion

It is genuinely believed that the Lamba retinal differentiation protocol works best with the cell line it was developed with. This was clearly demonstrated when MSUH001-iPSCs and Shef3-hESCs failed to achieve the similar retinal differentiation efficiency to H1-hESCs. Furthermore, as the differentiation efficiency was relatively low for these two cell lines, it requires a generic method that is applicable to a wide range of cell lines. Recent studies have shown that lowering O_2 tension has generic benefits in enhancing the production of specific cell types from human pluripotent stem cells (see section 1.5). In the next chapter, the effect of lowered O_2 tension will be examined on retinal differentiation of MSHU001-iPSCs and Shef3-hESCs.

5. The effect of hypoxia on the generation of RPCs from iPSCs and hESCs

5.1. Introduction

For human pluripotent stem cells to become clinically viable, it is critical to efficiently differentiate these cells into the desired cell types. Current strategies to induce differentiation into a particular lineage have tended to rely on mimicking the events that take place during the early mammalian development through the addition of pathway agonists and antagonists (Vazin and Freed, 2010). Differentiating cells are exposed to atmospheric oxygen (O_2) tension (~20%), which is significantly higher than the hypoxic environment experienced by developing human (~3%) and mammalian embryos (rabbit 8.7% and monkey 1.5%) (Fischer and Barister, 1994; Burton and Jaunaiux, 2001). Recent studies have shown that mimicking physiological O_2 tension can greatly influence embryonic stem cell (ESC) biology. Culturing human ESCs (hESCs) at 3-5% O_2 resulted in better maintenance of pluripotency marker expression, reduced levels of spontaneous differentiation and a decrease in the frequency of chromosomal abnormalities (Ezashi et al., 2005; Forsyth et al., 2006; Zachar et al., 2010). It has also been reported that low O_2 enhances endothelial, chondrogenic and neuronal differentiation of hESCs and neural and cardiac differentiation of mouse ESCs (mESC) (see section 1.4.3).

A number of studies have also mimicked O_2 tensions present in adult organs during the culture of adult stem cells. Low O_2 tension in the brain is conserved amongst mammalian species. In the rat brain, O_2 tension varies at different locations (white cortex 0.8%-2.1%, hypothalamus 1.4%-2.1% and hippocampus 2.6%) (Erecinska and Silver, 2001) as with human brain (3.2% under and 4.4% above the dura) (Ding et al., 1998). The subventricular zone (SVZ) is the main neurogenic niche in the human brain where neural stem cells (NSCs) are responsible for adult neurogenesis (Lim and Alvarez-Buylla, 1998). The O_2 tension of the human SVZ has never been directly measured however, it is widely accepted that NSCs reside in areas of relatively low O_2 tension. This is further supported by studies showing that human NSCs showed improved survival, proliferation and maintenance of an undifferentiated phenotype when cultured at 5% O_2 tension (Santilli et al., 2011). Low O_2 tensions also exist in other adult stem cell niches such as bone marrow, a source of hematopoietic (HSCs) and mesenchymal stem cells (MSCs) (Mohyeldin et al., 2010). Indeed, there is evidence that lower O_2 tension promotes the maintenance of an undifferentiated state in cultured bone marrow-derived MSCs (Cipolleschi et al., 1995; Fehrer et al., 2007). Taken together these studies have revealed that mimicking in-vivo O_2 tensions can be used to enhance the undifferentiated expansion and directed differentiation of both pluripotent and adult stem cells.

5.2. Aim

In this study, it was sought to improve the differentiation of human pluripotent stem cells into retinal progenitor cells (RPCs) by lowering O_2 tension. A previously reported retinal differentiation protocol (see chapter 4) was used to examine the effect of lowering O_2 tension on early neural retinal differentiation of iPSCs and hESCs. It was previously shown that 2% O_2 tension increases neuronal differentiation efficiency of mESCs (Mondragon-Teran et al., 2009). This O_2 tension is used during retinal differentiation of the two cell lines to observe whether low efficiency observed in the previous chapter could be enhanced to produce higher percentage of Pax6 and Chx10 co-expressing RPCs.

5.3. Detection of hypoxia in cells exposed to 2% O_2

Pimonidazole hydrochloride is a widely used hypoxic marker for detection of hypoxia both in-vitro and in-vivo due to its chemical stability and solubility in water (Raleigh et al., 1985). In the cells exposed to low O_2 tension, pimonidazole hydrochloride forms adducts with thiol groups in proteins whose immunoreactive side chain can be immunochemically analyzed using a monoclonal antibody raised against the adducts (Cline et al., 1990). Two human pluripotent stem cell lines were cultured at 2% O_2 for two days in the hypoxic chamber stained positively for hypoxyprobe-1 whereas those cultured at 20% O_2 were negative (Fig 5.1E-F). The exposure of human cells to O_2 tensions below 21% O_2 leads to the stabilization of HIF-1a and the activation of over 250 downstream genes including vascular endothelial growth factor (VEGF) and lactate dehydrogenase (LDHA) (Ke and Costa, 2006). We next investigated whether 2% O_2 had led to the expression of HIF-1a responsive gene VEGF in human pluripotent stem cells exposed to 2% O_2 for two days. The analysis revealed a similar fold increase in the expression level of VEGF in both iPSCs and hESCs (2.2 and 2.3 fold increase respectively, $p \leq 0.05$, Fig 5.1G and H) exposed to 2% O_2 compared to cells cultured under 21% O_2. This provides supporting evidence cells cultured in the hypoxic chambers at 2% O_2 (Mondragon-Teran et al., 2009) were exposed to hypoxic conditions.

Figure 5.1

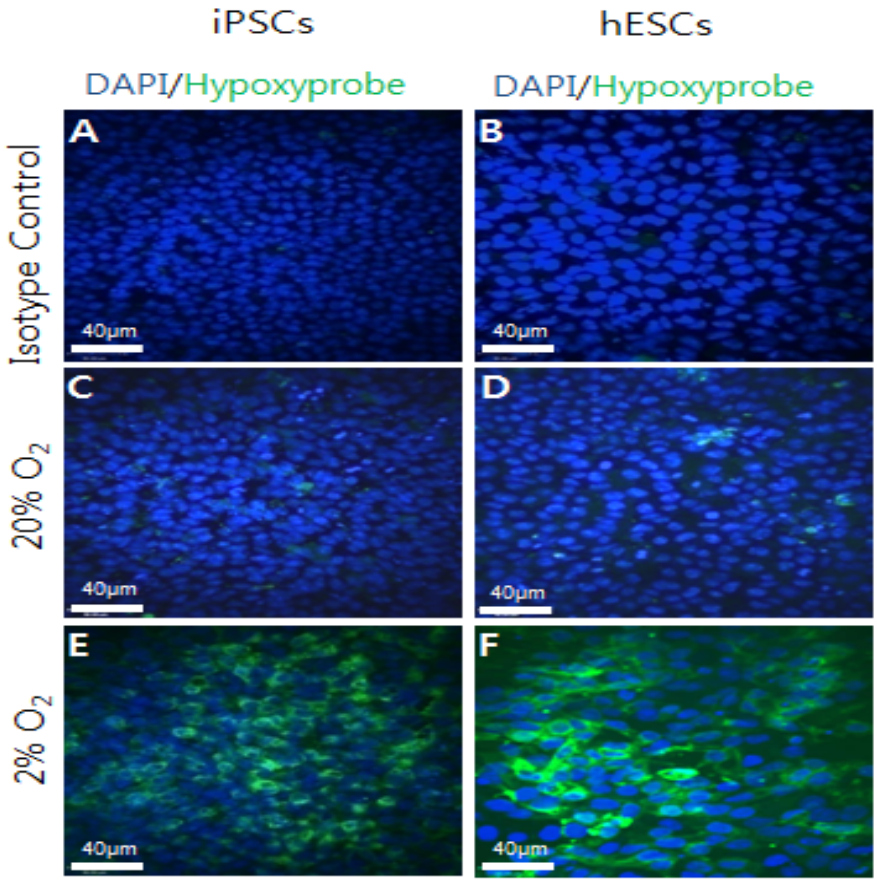


Figure 5.1 Validation of the hypoxic chamber. Confocal analysis of hypoxia marker in the cells exposed to 2% O_2. (A and B) Isotype control and (C and D) cells cultured at 20% O_2 for two days stained negatively for pimonidazole staining. (E and F) Cells cultured at 2% O_2 for two days however, stained positively for pimonidazole.

Figure 5.2

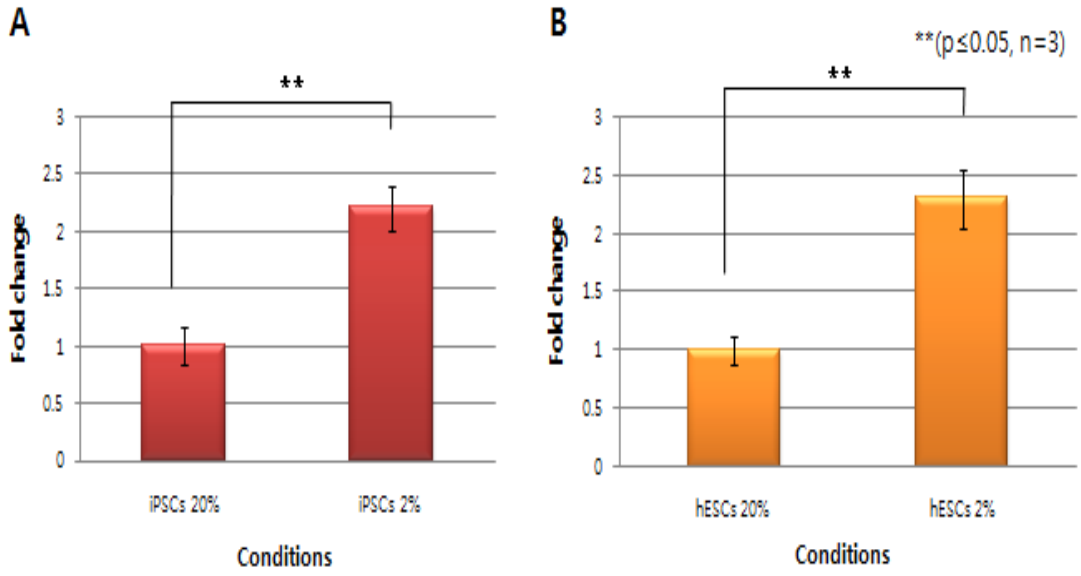


Figure 5.2. The activation of HIF-1a target gene VEGF. iPSCs and hESCs were cultured in the chamber for 6 days and qPCR was performed. The analysis revealed a significant 2.3 fold increased in the expression level of VEGF in both (A) iPSCs and (B) hESCs cultured in the chamber compared to those cultured at 20 % O_2.

5.4. Activation of HIF responsive genes and retinal specification in EBs derived from iPSCs (iPSCs-EBs)

Clinical application of ESCs-based cell therapy is often perceived as unethical due to the use of embryos and has drawbacks such as immune rejection. iPSC based cell therapies would circumvent the use of embryos and may minimise the risk of immune rejection through treating patients with their own cells (Rolletschek and Wobus, 2009). Therefore we examined whether lowering O_2 tension had a beneficial impact on the early neural retinal differentiation of iPSCs. EB formation is a widely used technique in the initial stage of pluripotent stem cell differentiation (Thomson et al., 1998). iPSCs colonies were dissected into small clumps and cultured in suspension for 3 days to form EBs at 20% and 2% O_2 in the presence of eye field inducing factors (Fig 2.1B and D). The expression level of pluripotency genes in EBs formed under both O_2 tensions was comparable to that of pluripotent iPSCs (Fig. 5.3A). Although qPCR analysis of Oct4 and Nanog revealed that their expression was lower at 2% compared to 20% O_2, these results were not significant. Immunocytochemistry of D3 EBs revealed that many cells formed in both conditions remained Oct4-positive (Fig. 5.3B) indicting that 2% O_2 did not affect the down-regulation of pluripotency markers in EBs differentiated in the presence of DKK-1, Noggin and IGF-1.

The exposure of human cells to O_2 tensions below 21% O_2 leads to the

stabilization of HIF-1a and the activation of over 250 downstream genes including vascular endothelial growth factor (VEGF) and lactate dehydrogenase (LDHA) (Ke and Costa, 2006). We next investigated whether 2% O_2 had led to the expression of HIF-1a responsive genes VEGF and LDHA in EBs exposed to 2% O_2 for 3 days. qPCR analysis showed a significant 3.2- fold increase in VEGF expression and a 4.8-fold increase in LDHA expression in EBs formed at 2% O_2 compared to 20% O_2 ($p \leq 0.05$ in both cases, Fig. 5.4A and B). The results confirm that EBs cultured at 2% O_2 for 3 days in the presence DKK-1, Noggin and IGF led to the up-regulation of HIF-1a responsive genes, most probably through O_2 dependent stabilization of HIF-1a.

Next, to determine whether lowered O_2 tension acts in synergy with the growth factors to induce the retinal specification of pluripotent cells, we performed qPCR on D3 EBs to determine the expression level of Six3 and Lhx2 which are two genes required for early eye field formation (Tetreault et al., 2009; Lie et al., 2010). The analysis revealed that there was a 2.2 fold increase ($p \leq 0.05$) in Six3 expression when EBs were differentiated at 20% O_2 in the presence of DKK-1, Noggin and IGF-1 compared to spontaneously differentiating EBs controls at 20% O_2 in the absence of any growth factors (Fig. 5.5A). However, we observed a bigger margin of increase in the expression level of Six3 when EBs differentiated with all growth factors at 2% O_2 compared to spontaneously differentiating EBs at 20% O_2 (3.1 fold increase, $p \leq$

0.05, Fig. 5.5A). We observed a similar phenomenon with Lhx2 expression level. The expression level of Lhx2 in EBs differentiated with growth factors at 20% O_2 was 1.2 fold higher than that in the spontaneously differentiating EBs (not significant, $p>0.05$, Fig. 5.5B). However, Lhx2 expression level significantly increased when EBs differentiated in the presence of all growth factors at 2% O_2 compared to spontaneously differentiating EBs at 20% O_2 (2.5 fold increase, $p\leq0.05$, Fig.5.5B).

Figure 5.3

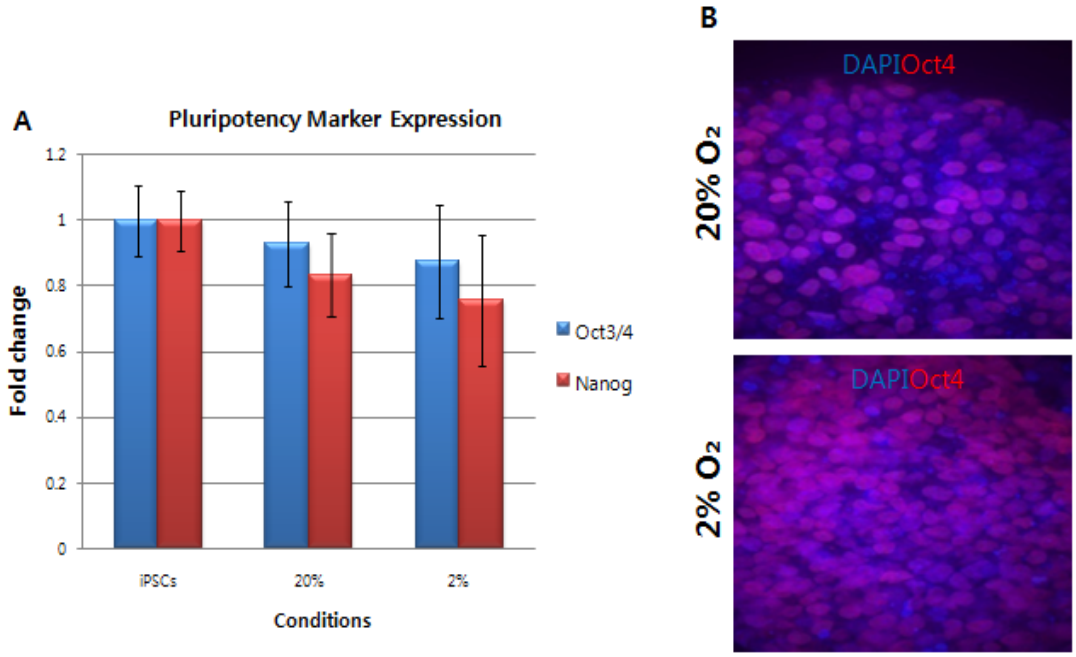


Figure 5.3 Expression of pluripotency genes in D3 iPSCs-EBs cultured in both O_2 conditions. (A) Differentiating iPSCs-EBs in the presence of the growth factors for 3 days in both O_2 conditions showed a comparable expression level of pluripotency genes to the control pluripotent iPSCs. (B) Confocal microscopy revealed that iPSCs-EBs formed in both O_2 conditions in the presence of the growth factors were aggregates of Oct4-positive cells after 3 days of suspension culture. The blue and red bar represents the expression level of Oct4 and Nanog respectively.

Figure 5.4

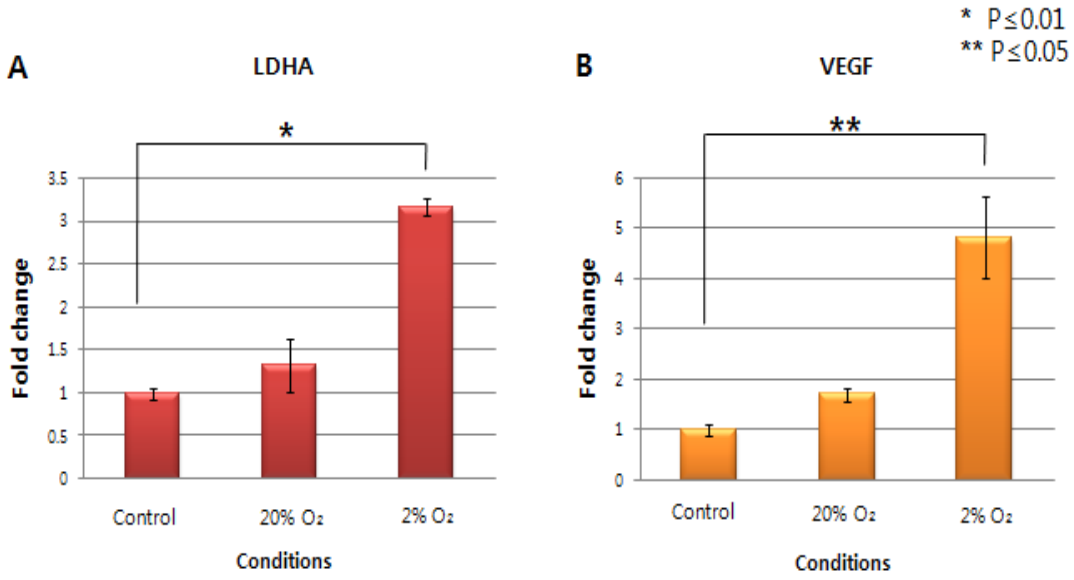


Figure 5.4. Relative expression level of HIF-1aresponsive genes. (A and B) qPCR analysis of LDHA and VEGF expression level in iPSCs-EBs formed at 2% compared to 20% O_2 in the presence of the growth factors.

Figure 5.5

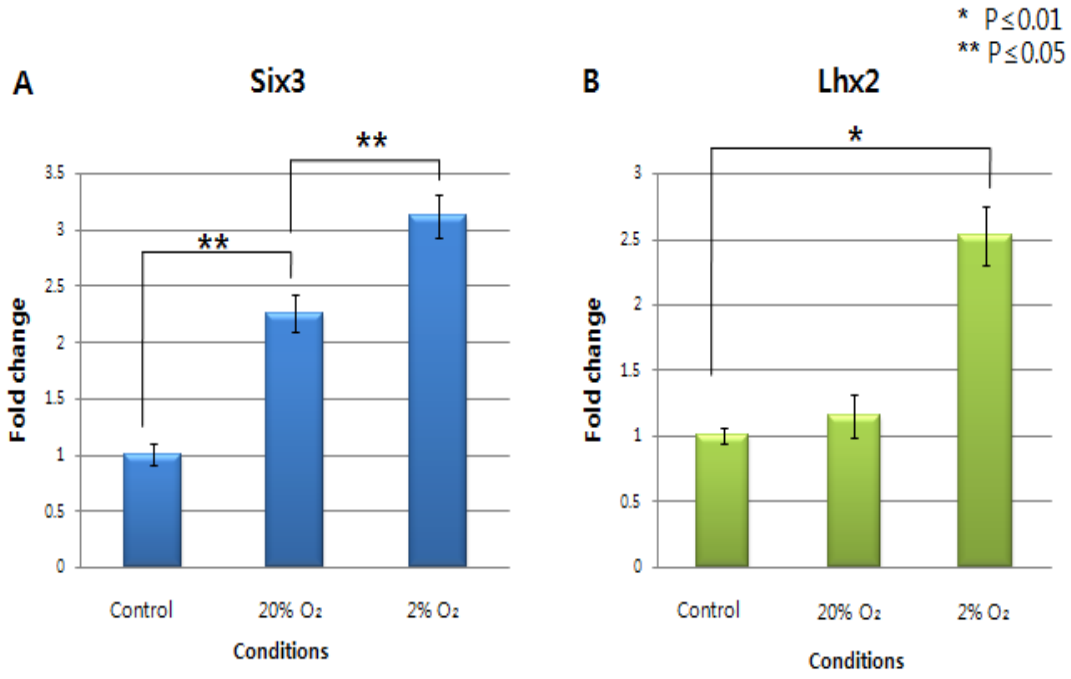


Figure 5.5 Relative expression level of early eye field genes Six3 and Lhx2. (A and B) qPCR analysis of the gene expression levels in iPSCs-EBs differentiated in the presence of growth factors at 20% and 2% O_2 relative to spontaneously differentiating control iPSCs-EBs at 20% O_2.

5.5. Effect of lowered O_2 tension on generation of RPCs derived from iPSCs (iPSCs-RPCs)

3 days after plating EBs, cells migrated as tightly packed neuroepithelial cells from the site at which the EBs had attached under both conditions in a similar pattern as previously observed (Figure 5.6B) (Meyer et al., 2009). 8 days after plating EBs, densely-packed populations of cells arranged in a radial manner, termed as neural rosettes due to their resemblance to early neural tube development (Zhang et al., 2001) formed in close proximity to the original site of attachment at 2% O_2 (Fig 5.6B, arrows). At 20% O_2 neural rosettes did not form until 10 days after plating (Fig 5.6A). The morphology of cells under both conditions became multilayered and indistinguishable 17 days after plating EBs (Fig 5.6A and B).

Pax6 and Chx10 are key transcription factors required for RPC multipotency and proliferation (Liu et al., 1994; Marquardt et al., 2001) and it has been shown that cells co-expressing these markers can be generated from human pluripotent stem cells 21 days after plating EBs in the presence of DKK-1, Noggin and IGF-1 (Lamba et al., 2006) (see chapter 4). Using immunocytochemistry, we found that cells co-expressing RPC markers Pax6 and Chx10 were visually more abundant at 2% O_2 relative to 20% O_2 21 days after plating EBs (Fig 5.7A and B). At 20% O_2, the areas of Pax6 positive cells were abundant throughout the culture but only a few colonies co-expressed Chx10 (Fig 5.7A). Pax6 positive cells were also abundant in the culture

at 2% O_2 (Fig 5.7) but more colonies co-expressing Pax6 and Chx10 were observed at 2% O_2 as compared to 20% O_2 (Fig 5.7B).

Figure 5.6

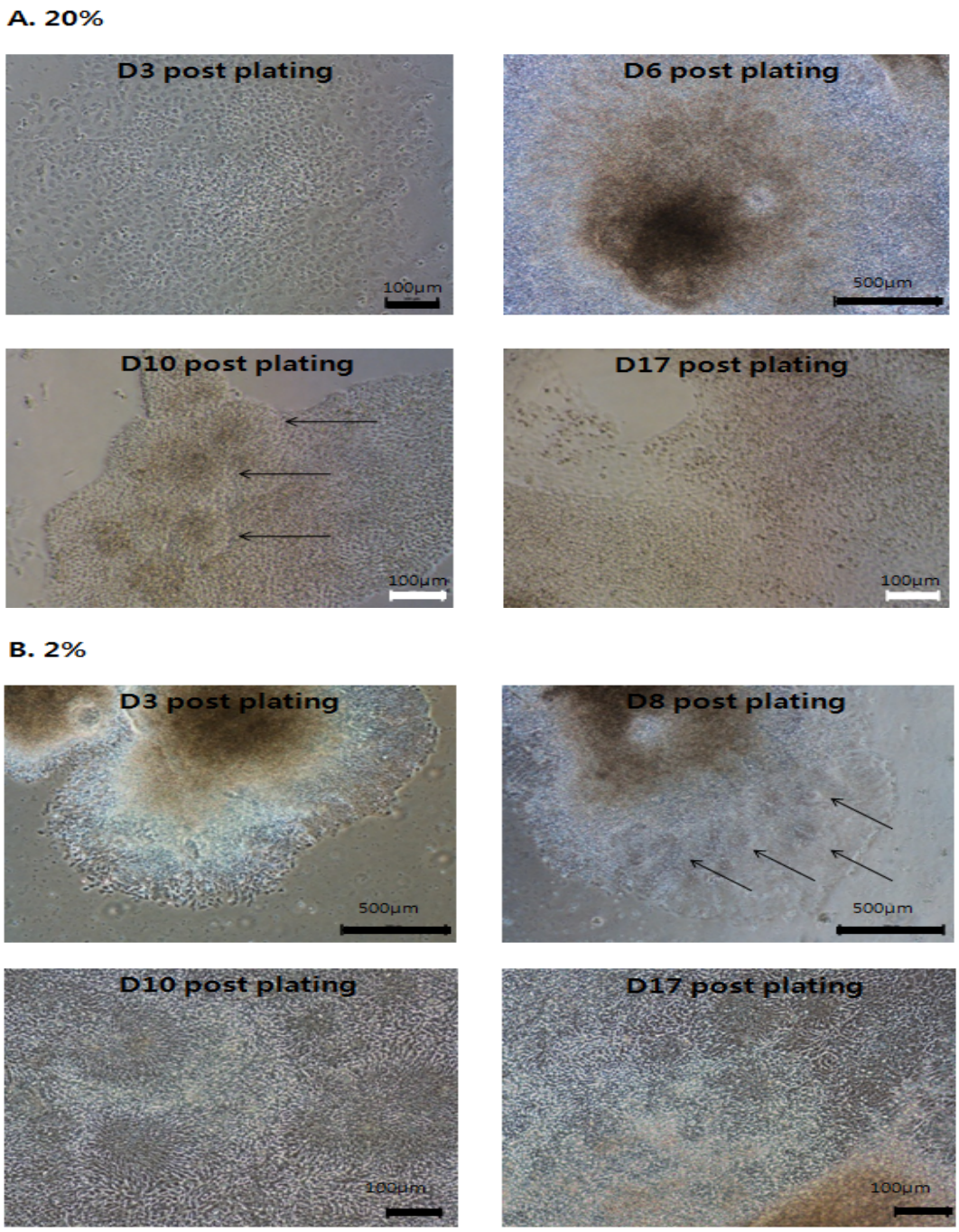


Figure 5.6 Generation of RPCs from iPSCs at 20% and 2% O_2. (A) Morphological analysis retinal differentiation of iPSCs at 20% O_2 tensions. Representative images were taken on D3, D6, D10 and D17 after plating iPSCs-EBs.(B) Morphological analysis of iPSCs-EBs at 2% O_2. Representative images were taken on D3, D8, D10 and D17 after plating iPSCs-EBs. Arrows indicate neural rosette formation. Neural rosettes were formed 10 and 8 days post plating EBs at 20% and 2% O_2 respectively..

Figure 5.7

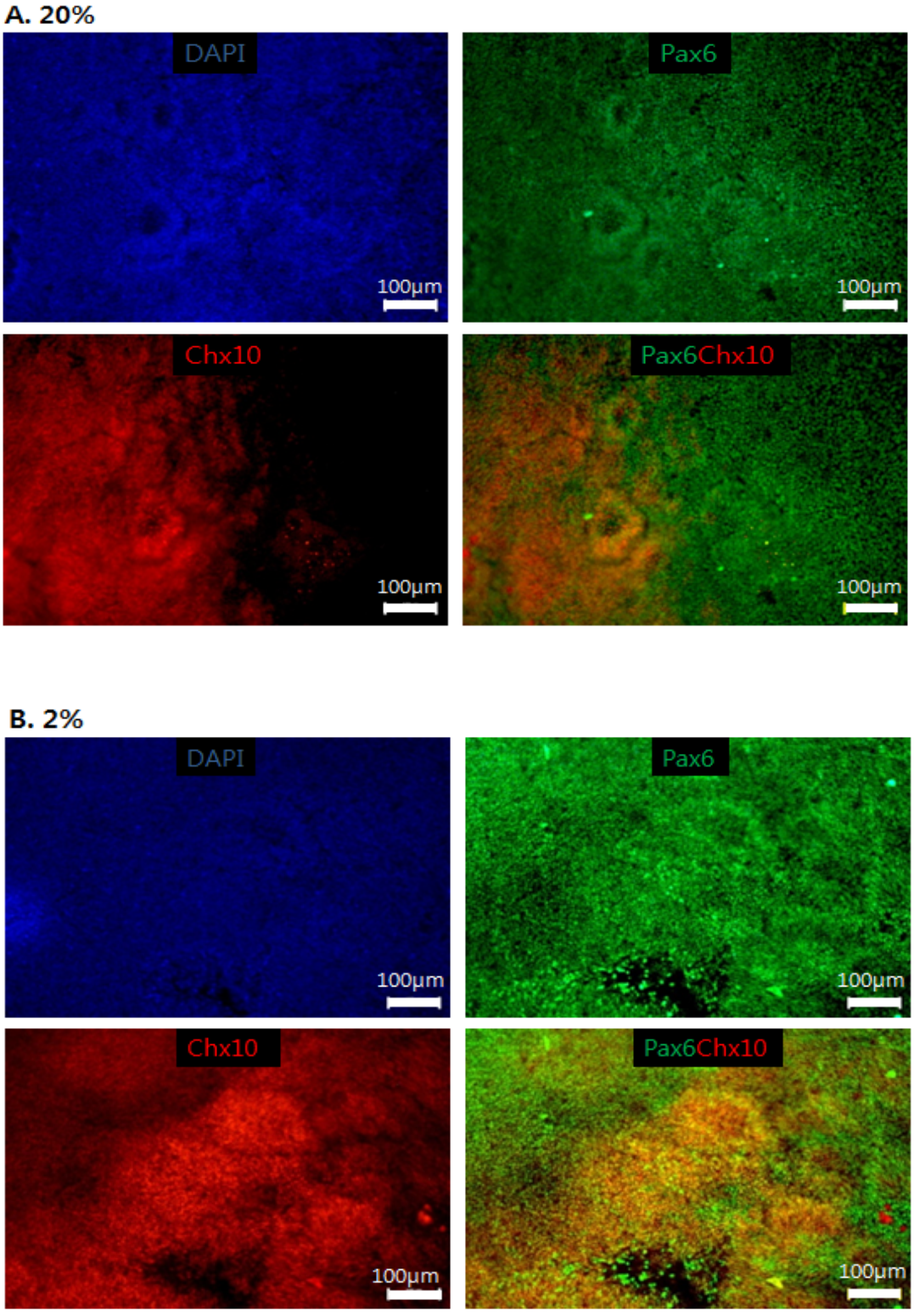


Figure 5.7 Representative images of colonies co-expressing Pax6 and Chx10 at 20% O_2 (A) and 2% O_2 (B).

Quantification of iPSCs-RPCs using flow cytometry revealed that at 20% O_2, 74±4.6% were Pax6 positive and 31±2.8% of the culture expressed chx10 21 days after plating EBs (Fig 5.8 A and B). At 2% O_2, there was a slight increase in the percentage of cells expressing Pax6 (80±3.8%) although this rise was not found to be significant ($p>0.05$ Fig 5.8C). Culture at 2% O_2 was associated with significant rises in the percentage of cells expressing Chx10 (55±3.7% $p\leq0.05$, Fig 5.8D). The majority of these Chx10 expressing cells also expressed Pax6 and their percentage also significantly increased at 2% compared to 20% O_2 (54±1.3% $p\leq0.05$, Fig 5.9A and B). These results confirm our earlier observation using immunocytochemistry and quantify the improvements in RPC formation which can be achieved at 2% O_2. At the same time point, cells expressing other retinal markers such as Otx2, Lhx2 and Six3 were also observed by immunocytochemistry but no visible differences were found between the two conditions (Fig 5.10).

Figure 5.8

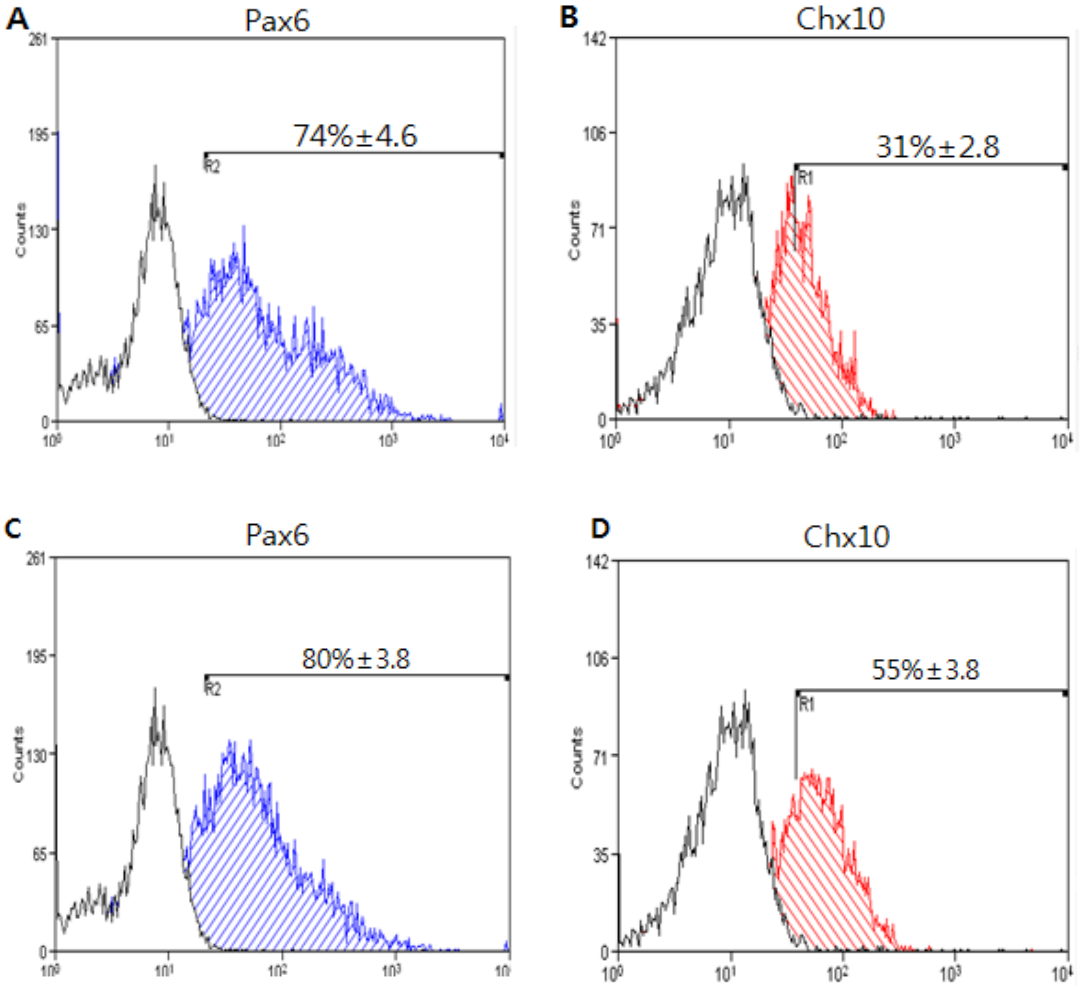


Figure 5.8 Comparison between RPC generation at 20 and 2% O_2. (A and B) Flow cytometry analysis of Pax6 and Chx10 expression level at 20% O_2. (C and D) Flow cytometry for Pax6 and Chx10 expression level at 2% O_2. Each histogram represents a population stained with the corresponding isotype and secondary antibody (blank) and with primary.

Figure 5.9

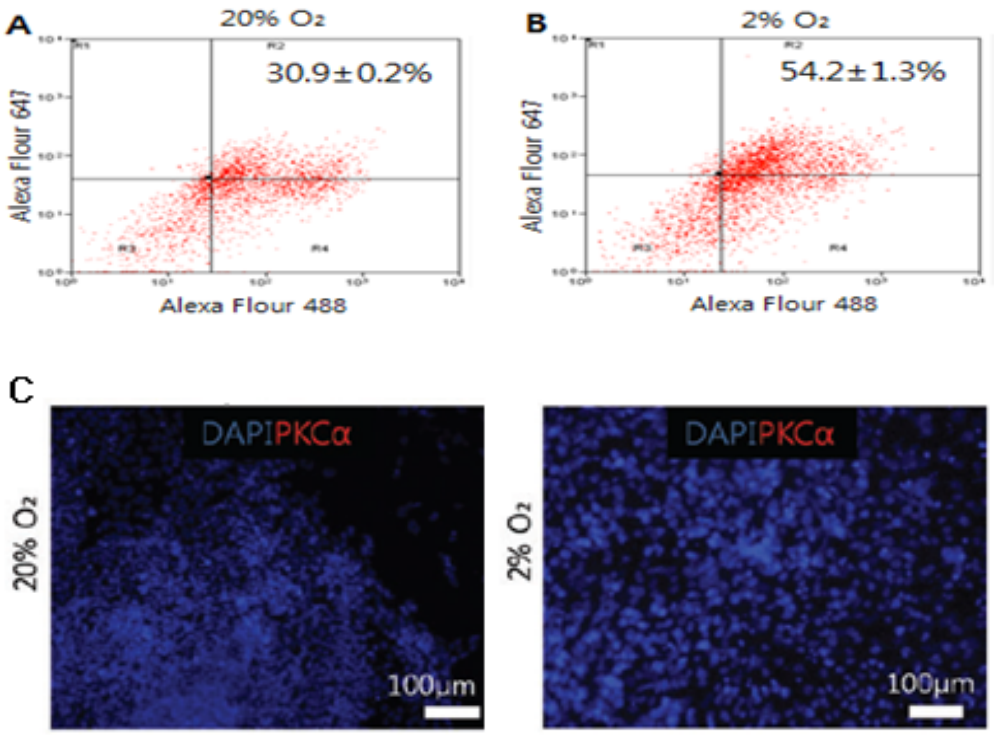


Fig 5.9. (A and B) Dot plots revealed that the majority of cells expressing Chx10 also expressed Pax6, thus represent a true RPC population. (C) Immunucytochemistry was performed with an antibody raised against bipolar marker PKCα at the end of D21 post plating EBs at both O_2 tensions. No immunoactivity against PCKα meant that Chx10 expression is RPCs.

Figure 5.10

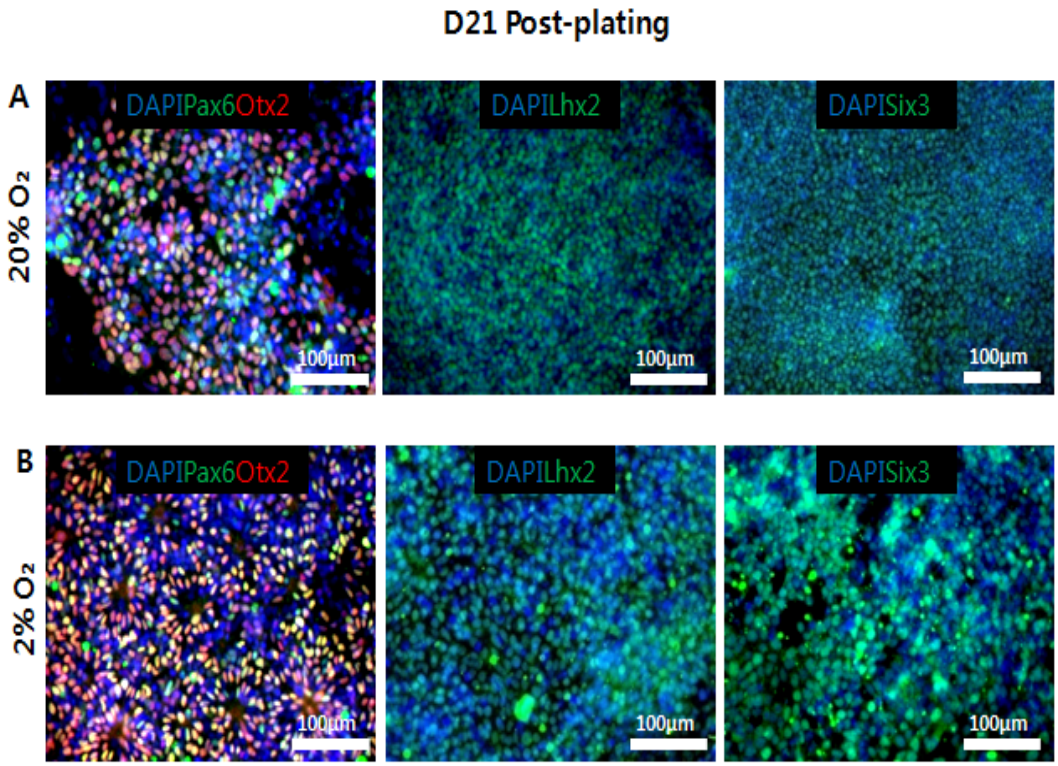


Figure 5.10. Immunocytochemistry for other retinal markers. (A and B) Immuocytochemistry was performed with antibodies against Pax6, Otx2, Six3 and Lhx2 at 20% and 2% O_2 respectively. In accordance with qPCR data, these markers were also detectable at the protein level and no significant differences were observed between the two conditions.

21 days after plating EBs, our qPCR analysis revealed no significant increases in the expression levels of early eye field markers such as Lhx2, Otx2, Six3 and Rx at 2% vs 20% O_2 ($p>0.05$ in all cases). However, a significant 1.2- and 3.8-fold increase in the expression level of Pax6 and Chx10 at 2% O_2 respectively relative to 20% O_2 (Fig 5.11). This does not however indicate that at 2% O_2 culture, many Chx10 positive cells are not co-expressing Pax6. As seen before with flow cytometry data (Fig 5.8), there is a small difference in the expression level of Pax6 between the two conditions and much larger increase in the expression of Chx10. As we revealed all the Chx10-positive cells co-expressed Pax6 (Fig 5.9A and B), we can now confidently say that an increase in the expression level of Chx10 corresponds to Pax6/Chx10 co-expressing RPC populations. This was further supported when we detected no immunoactivity to an antibody raised against PKCα 21 days after plating EBs in both conditions as Chx10 is also a marker for mature bipolar cells (Fig 5.9C). However, at the same time point, we showed the expression of Neurofilament-M (a marker for ganglion cells) and Prox-1 (a marker for horizontal cells) in both conditions (Fig 5.12). This coincides well with the early retinal development as RPCs sequentially differentiate into ganglion, cones and horizontal cells first, then rods and amacrine cells followed by muller glial and bipolar cells. We failed to detect PKCα and markers for mature photoreceptors (S-opsin and Rhodopsin) may signify

that retinal differentiation was prematurely terminated 21 days after plating EBs. However, we detected a small number of cells co-expressing Crx (a cone precursor maker) and IRBP (interphotoreceptor retinoid binding protein) 21 days after plating EBs at 2% O_2 (Fig 5.12). These cells were however, not found in 20% O_2 culture, carefully suggesting that lowered O_2 may favour the development of cone photoreceptors over other types of retinal neurons. The timing of the appearance of Crx/IRBP positive cells also correlates well with the early retinal development as cones are the first retinal neuron to be generated along with ganglion and horizontal cells from RPCs. The inability of lowered O_2 to significantly increase the expression level of these late retinal markers suggests that lowered O_2 is selectively acting on the early stages of retinal differentiation.

Figure 5.11

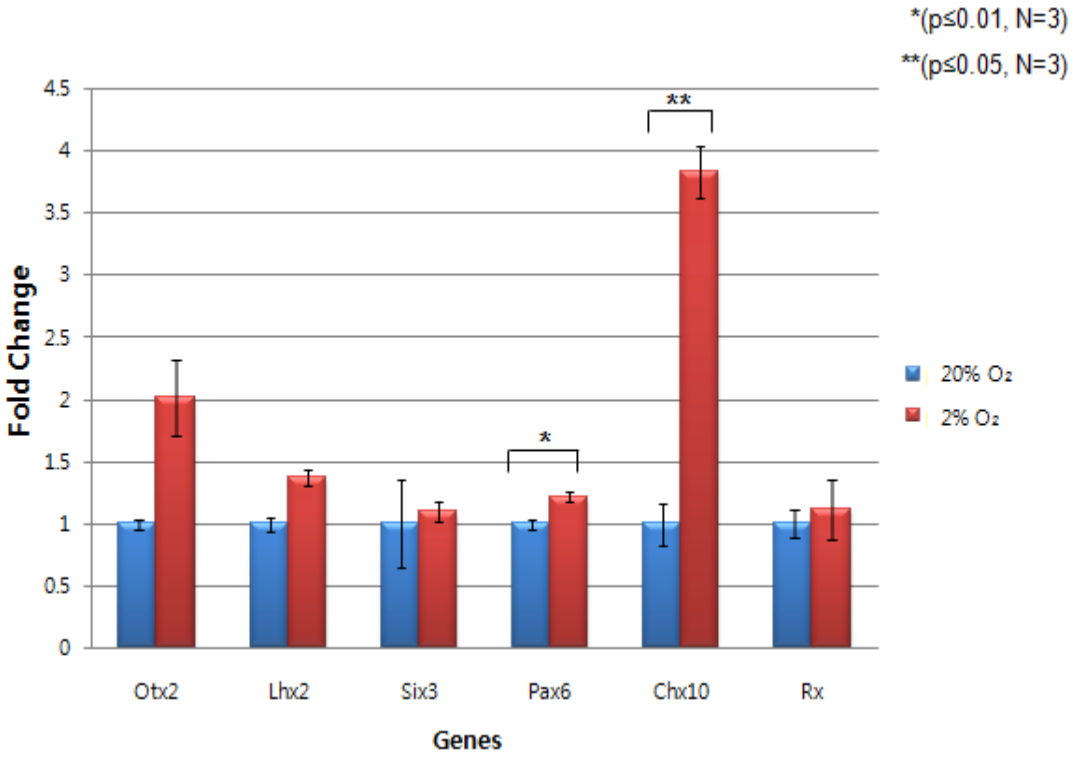


Figure 5.11.Relative mRNA expression of the retinal markers 21 days after plating iPSCs-EBs at 20% and 2% O_2. Compared to the control 20% O_2 differentiation, 2% O_2 induced an increase in the expression level of the early eye field genes (Otx2, Lhx2, Six3 and Rx) but without any statistical significance ($p>0.05$). However, a significant increase was observed in the expression level of RPC markers Pax6 and Chx10 under 2% compared to 20% O_2 culture ($p\leq0.05$ for Pax6 and $p\leq0.01$ for Chx10, n=3). The blue and red bar represents retinal differentiation of MSUH001-iPSCs at 20% and 2% O_2 respectively 21 days after plating EBs.

Figure 5.12

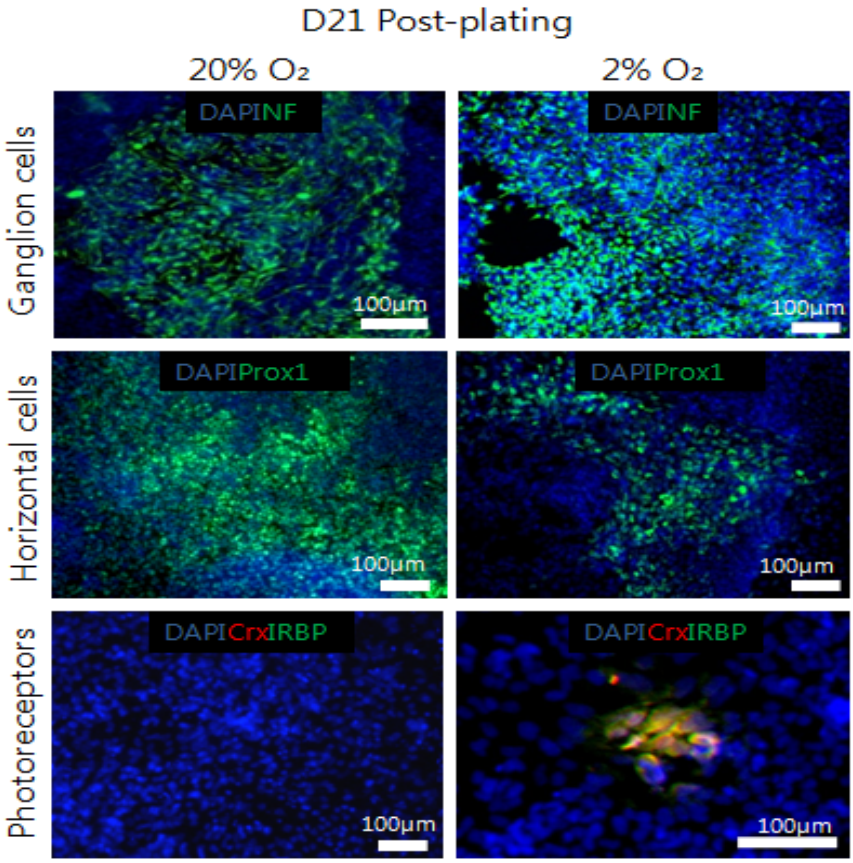


Figure 5.12. Multilineage differentiation of iPSCs-RPCs at 20% and 2% O_2. Immunocytochemistry to antibodies raised against ganglion (Neurofilament-M), horizontal (Prox1) and photoreceptor (Crx and IRBP) makers at both conditions.

5.6 Generation of RPCs from hESCs (hESCs-RPCs) under 20% and 2% O_2

hESCs colonies were removed from the feeder layer and cultured in suspension in the presence of the same growth factors for 3 days in both O_2 tensions (Fig. 2.1 B). In line with iPSCs-EBs, no significant down-regulation of pluripotency gene expressions was observed in EBs cultured at both O_2 tensions compared to spontaneously differentiating EBs at 20% O_2 (Fig 5.13A). In addition, most of cells in EBs expressed Oct4 in both O_2 tensions (Fig. 5.13B). It was observed that a significant 2.1 fold increase ($p \leq 0.05$) in the expression level of HIF-1a target gene VEGF in EBs cultured at 2% compared to 20% O_2 (Fig. 5.14).

3 days after plating hESCs-EBs at 2% O_2, the cultures contained spindle-like cells which formed extensive neurite networks on the periphery of the attached EBs (Fig. 5.15, arrows). Although the same spindle cells were visible at 20% O_2, there appeared to be fewer neurite processes in comparison to 2% O_2 culture (Fig. 5.15A and B, arrows). Neural rosettes were formed 8 days after plating EBs at 2% O_2 which did not appear until 12 days after plating EBs at 20% O_2 (Fig. 5.15A and B, asterisks). 18 days after plating EBs the morphology became increasingly difficult to define using phase contrast microscopy as cells became multilayered in both O_2 conditions.

Figure 5.13

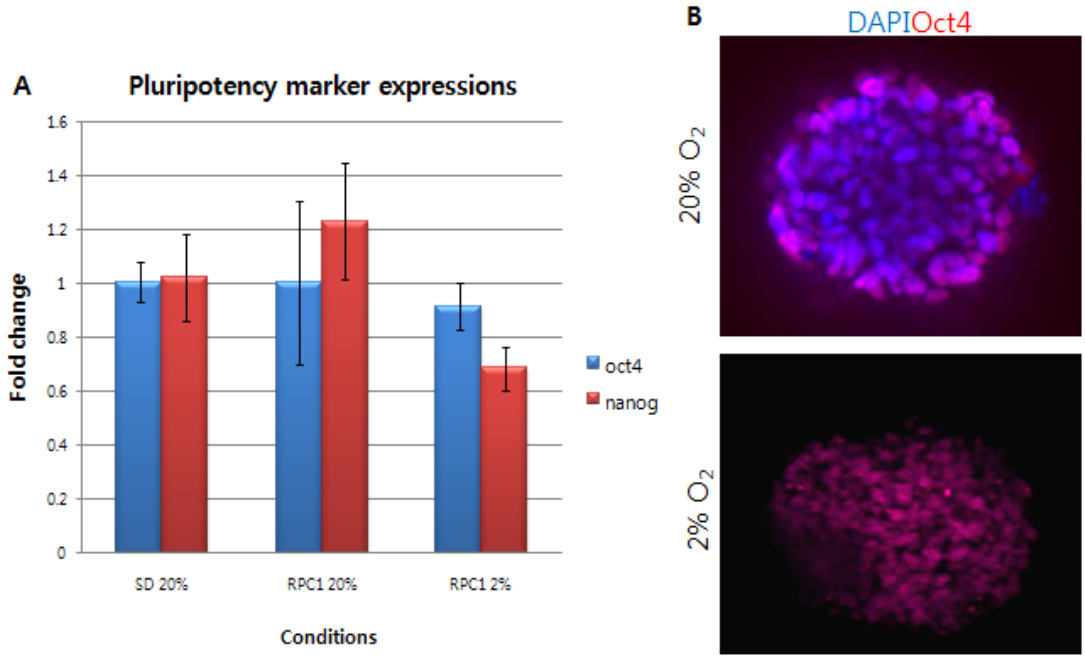


Figure 5.13 Expression of pluripotency genes in D3 hESCs-EBs cultured in both O_2 conditions. (A) Differentiating hESCs-EBs in the presence of the growth factors for 3 days in both O_2 conditions showed a comparable expression level of pluripotency genes to the control pluripotent hESCs. (B) Confocal microscopy revealed that hESCs-EBs formed in both O_2 conditions in the presence of the growth factors were aggregates of Oct4-positive cells after 3 days of suspension culture. The blue and red bar represents the expression level of Oct4 and Nanog.

Figure 5.14

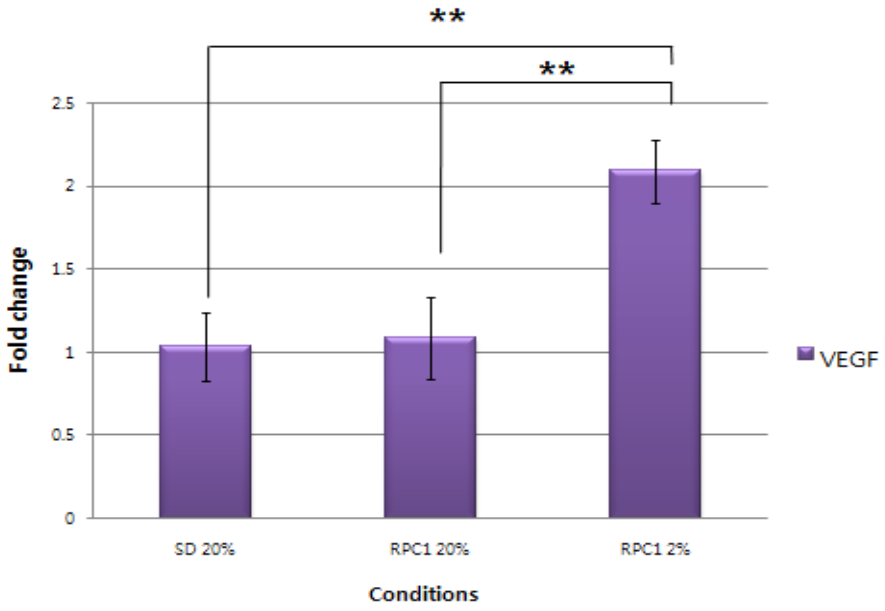


Fig 5.14. qPCR gene expression level analysis of HIF-1a responsive gene VEGF. Relative expression level of HIF-responsive gene VEGF in hESCs-EBs formed at 2% compared to 20% O_2 in the presence of the growth factors.

Figure 5.15

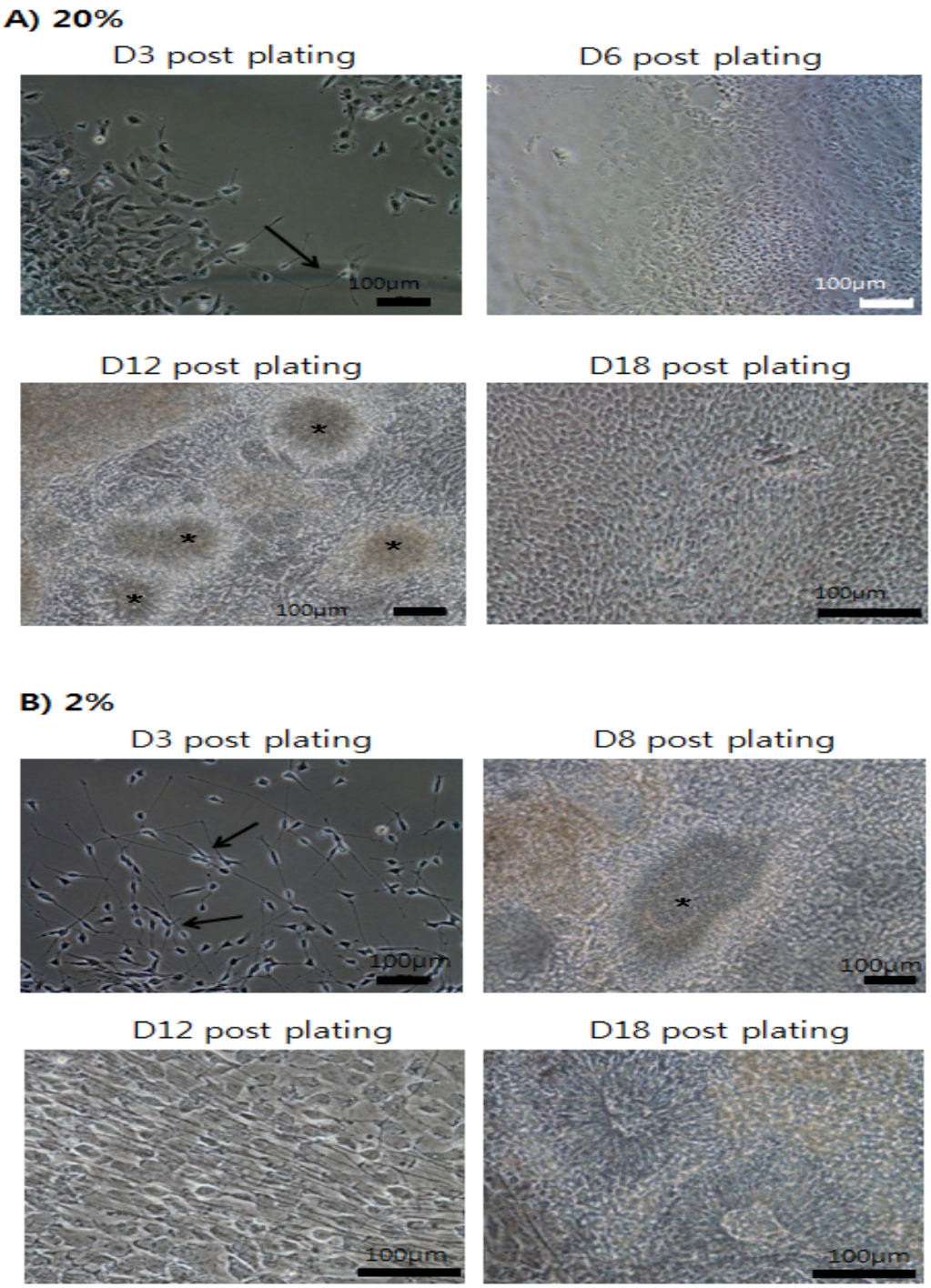


Figure 5.15 Generation of RPCs from hESCs at 20 and 2% O_2. (A) Morphological analysis retinal differentiation of iPSCs at 20% O_2 tensions. Representative images were taken on D3, D8, D12 and D18 after plating hESCs-EBs. (B) Morphological analysis of retinal differentiation at 2% O_2, images takens on D3, D8, D12 and D18 after plating hESCs-EBs. Arrows indicatene neuronal processes and Asterisks represent neural rosette formation 10 and 8 days post plating hESCs-EBs at 20% and 2% O_2 tensions respectively.

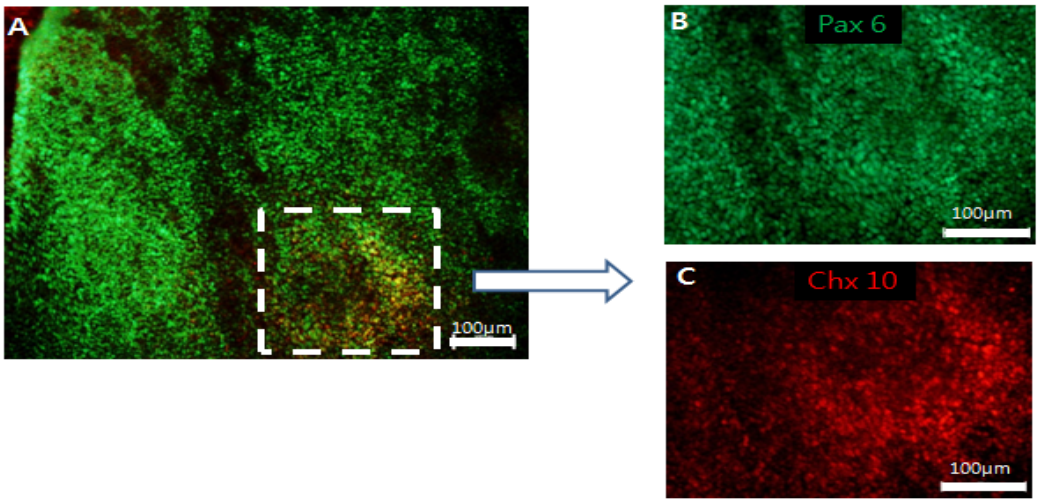
To examine the effect of lowered O_2 tension on early neural retinal differentiation of hESCs, immunocytochemistry was performed 21 days after plating EBs. At 20% O_2 there were large areas of Pax6-positive cells and only a small population of these cells co-expressed Chx10 confining to densely populated clusters of cells in close proximity to where EBs had attached (Fig. 5.16 A to C). At 2% O_2, Pax6 positive cells were also abundant but noticeably more of these cells co-expressed Chx10 (Fig. 5.16 D to G).

To quantify Pax6 and Chx10 co-expressing RPCs at both conditions, we used flow cytometry. This analysis revealed that at 2% O_2, significantly more cells expressed Pax6 (52±5.1%, $p \leq 0.05$) and Chx10 (24±5.1%, $p \leq 0.05$, Fig. 5.17B and C) when compared to 20% O_2 controls (Fig. 5.17A and BThese results confirm earlier observation using immunocytochemistry and quantify the improvements in RPC formation which can be achieved at 2% O_2.

Expression levels of early eye field and RPC markers wereobserved fourteen days after plating hESCs-EBs, the expression level of all the genes increased at 2% O_2 in comparison to 20% O_2 but only Pax6 and Chx10 were statistically significant ($P \leq 0.01$ and $p \leq 0.05$ respectively, Fig. 5.18), indicating that lowered O_2 tension enhanced transcription of RPC markers.

Figure 5.16

D21 post- plating at 20% O_2



D21 post-plating at 2% O_2

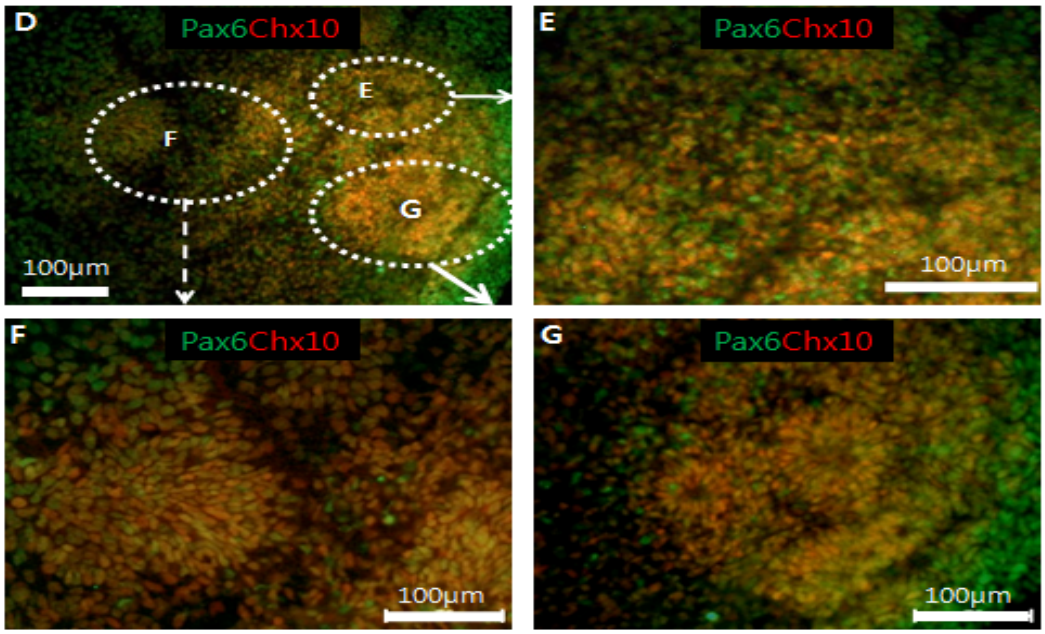


Figure 5.16. Generation of Pax6 and Chx10 co-expressing RPCs from hESCs at 2% O_2. Representative images of colonies co-expressing Pax6 and Chx10 at 2% O_2. (B-C) Higher magnification of colonies co-expressing Pax6 and Chx10. (E)

Figure 5.17

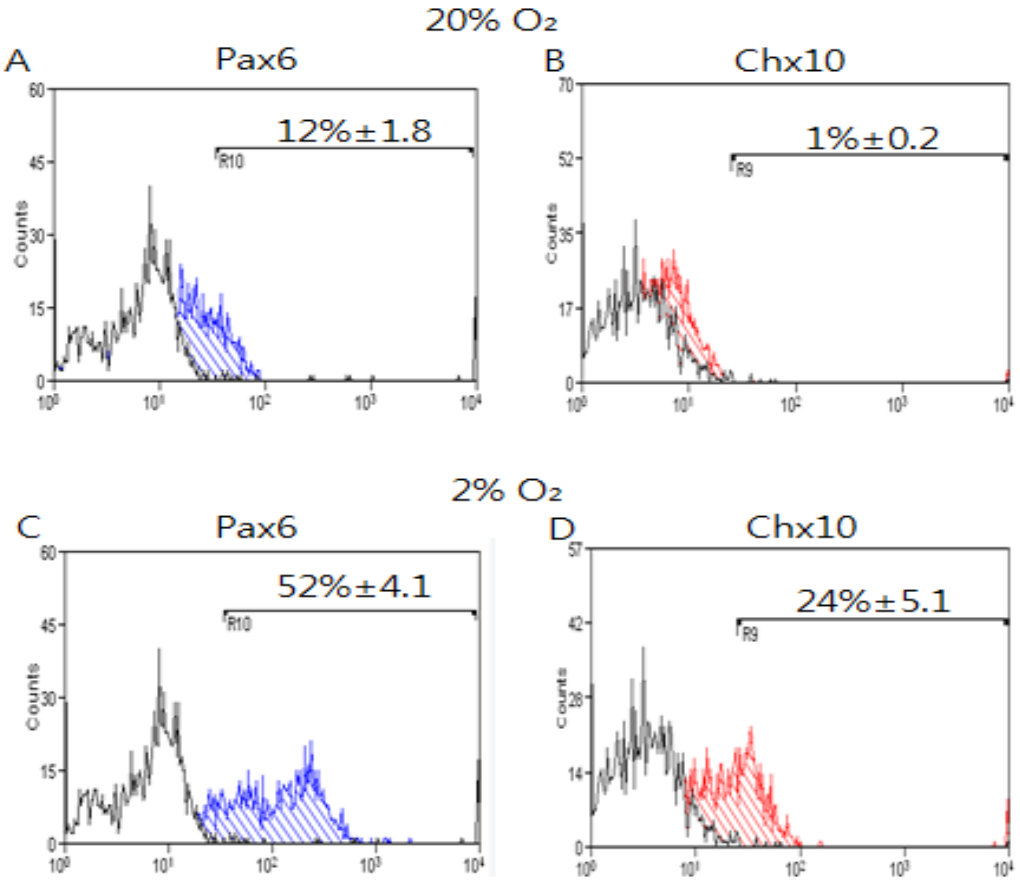


Figure 5.17. Comparison between RPC generation at 20% and 2% O_2 21 days after plating EBs. (A and B) Quantification using flow cytometry revealed that the expression of Pax6 and Chx10 increased at 20% O_2. (C and D) It was revealed that the expression of both markers increased when differentiation took place at 2% O_2. Each histogram represents a poplation of stained with the corresponding isotype and secondary antibody (Blank) and with primary and secondary (dashed). The standard error is shown as a result of three independent experiments (n=3).

Figure 5.18

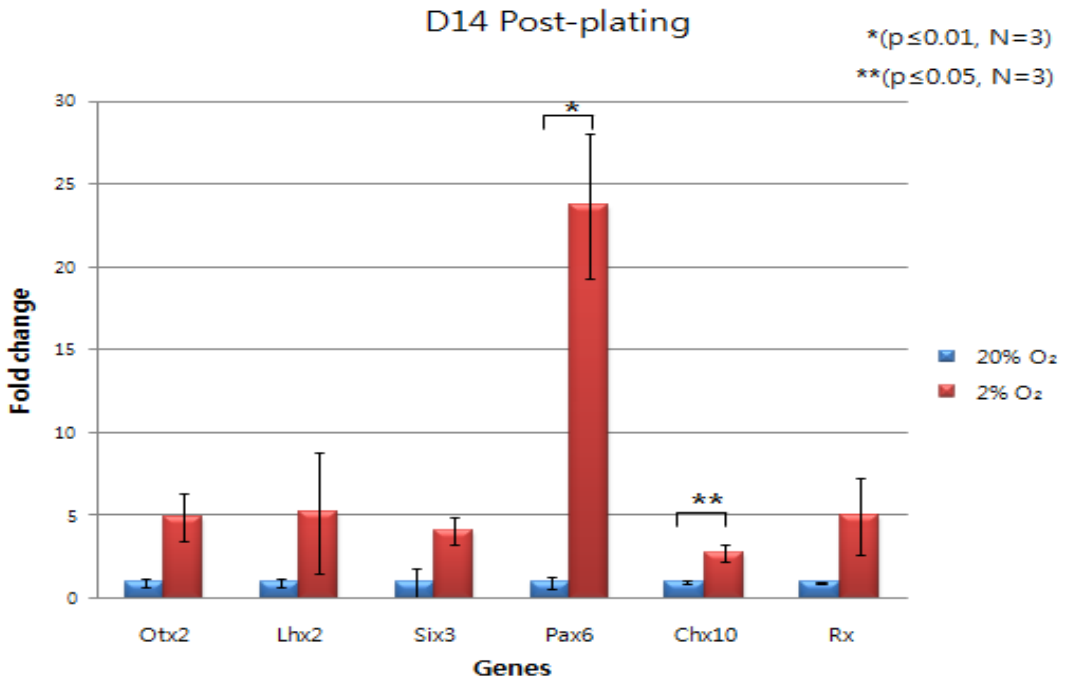


Fig 5.18. Quantitative analysis of relative mRNA expression of the early eye field and RPC markers of hESCs-RPCs. Statistically significant increase in the Pax6 and Chx10 mRNA expression was seen at 2% relative to 20% O_2 14 days after plating EBs ($p \leq 0.01$ for Pax6 and $p \leq 0.05$ for Chx10, n=3). The blue and red bar represents retinal differentiation of Shef3-hESCs at 20% and 2% O_2 respectively 21 days after plating EBs.

5.8. Discussion

In ocular genetic disorders, such as retinitis pigmentosa (RP), degeneration of photoreceptors leads to many forms of visual impairment from progressive vision loss to, in most severe cases, a complete blindness (Hartong et al., 2006). To date, these conditions remain untreatable. However efforts have been made to restore vision by means of transplanting post-mitotic photoreceptor precursors (McLaren et al., 2006; Lamba et al., 2010). Human pluripotent stem cells are an ideal source for generating transplantable retinal cells as they are capable of producing almost all types of cells in the body (Thomson et al., 1998). Using combinations of forebrain inducing growth factors such as DKK-1 (wnt pathway antagonist), Noggin (BMP pathway antagonist) and Lefty A (nodal pathway antagonist), it has been possible to generate RPCs from pluripotent stem cells with varying efficiencies (Lamba et al., 2006; Meyer et al., 2009; Osakada et al., 2008). In particular, Lamba et al. generated ~80% RPCs co-expressing RPC markers Pax6 and Chx10 21 days after plating hESCs-EBs (Lamba et al., 2006). In this present study, we have shown that the efficiency of this RPC differentiation protocol is cell line dependent and can be enhanced by lowering O_2 tension.

Organogenesis in mammalian embryos takes place in a low O_2 environment (~2-4% O_2) (Burton and Jaunaiux, 2001) and we have shown that mimicking this O_2 tension has a significant impact in inducing key retinal genes during differentiation.

Lhx2 and Six3 are early eye field genes required for retinal specification and optic cup development respectively (Tetreault et al., 2009; Liu et al., 2010). It has been reported that Lhx2 defines presumptive retina field and transactivates Six3 expression in the same domain (Tetreault et al., 2009). We have observed a non-significant increase in the expression level of Lhx2 in EBs differentiating at 20% O_2 in the presence of DKK-1, Noggin and IGF-1 compared to spontaneously differentiating EBs 20% O_2 ($p>0.05$ Fig 5.5B). However, the expression level significantly increased at 2% O_2 when EBs differentiated in the presence of the growth factors compared to spontaneously differentiating EBs at 20% O_2 (Fig 5.5B). The increase in the expression level of Lhx coincided with an increase in Six3 expression at 2% compared to 20% O_2 in the presence of the growth factors (Fig 5.5A), indicating that lowering O_2 to 2% acts synergistically with DKK-1, Noggin and IGF-1 to enhance the early retinal specification of iPSCs.

When iPSCs- and hESCs-EBs were plated for 21 days in both O_2 tensions, we observed the formation of neural rosette at an earlier time point at 2% O_2 compared to 20% O_2 (Fig 5.6 arrows and 5.16 asterisk). This suggests that 20% O_2 may delay the development of neural lineages cells from human pluripotent stem cells. In addition, there was a significant increase in the population of cells co-expressing Pax6 and Chx10 under 2% O_2 compared to 20% O_2 21 days after plating

iPSCs- and hESCs-EBs (Fig 5.8 and 5.17). Although the percentage of hESCs-RPCs was lower in comparison to iPSCs-RPCs, lowering O_2 tension increased the efficiency of RPC formation from both pluripotent cells types. Increasing differentiation efficiency of pluripotent stem cells in various lineages of cells under low O_2 (~2-5%) have been reported (Mondragon-Teran et al., 2009; Prado-Lopez et al., 2010; Stacpoole et al., 2011). In the case of neuronal differentiation, the efficiency was analysed by looking at pan neuronal markers such as BIII tubulin and MAP2 (Mondragon-Teran et al., 2009; Stacpoole et al., 2011). Here, we have shown that lowered O_2 tension could also be used to enhance generation of a specific population of progenitor cells in the central nervous system (CNS), which are capable of producing all six types of retinal neurons and glia cells (Young, 1985).

The exact mechanism of hypoxia on retinal differentiation requires further investigation but it has been reported that hypoxic conditions are critical for CNS development (Nyakas et al., 1996). Hypoxia inducible factor (HIF) pathway is activated by lower O_2 tension and studies of HIF knockout mice revealed embryonic lethality at E10.5 accompanied with neural tube defect and cell death within the cephalic mesenchyme (Iyer et al., 1998; Kotch et al., 1999). Vascular endothelial growth factor (VEGF) is a downstream target of HIF pathway (Marti and Risau, 1998; Shweiki et al., 1992). Although it is a well known growth factor for the

formation and maintenance of vascular structure (Ferrara et al., 2003; Grunewald et al., 2006), it also participates in neurogenesis via VEGFR2/Flk-1 receptor (Jin et al., 2002) and plays a significant role in developing the normal retina (Ishida et al., 2003; Stalmans et al., 2002). VEGFR2/Flk-1 is expressed in neural retina during mouse embryogenesis and has been reported that VEGF mediates mouse RPCs proliferation *in vitro* through VEGFR (Yang and Cepko, 1996). As low O_2 activates key signal pathway *in vivo* during early development, our results indicate that lowering O_2 tension during *in vitro* differentiation may have activated key signaling pathways required for CNS development.

Increasing the efficiency of RPC generation from human pluripotent stem cells has a significant advantage for the therapeutic application of stem cell therapy for genetic ocular disorders. It has been reported that RPCs generated from human pluripotent stem cells are common progenitor cells for Crx and Nrl expressing cells that are capable of forming functional photoreceptors upon transplantation (McLaren et al., 2006; Lamba et al., 2009; Lamba et al., 2010) However, the efficiency of producing these cells is very low and has not been optimized which hinders clinical application for treating blindness (Lamba et al., 2010). Our results demonstrate that lowering O_2 tension enhanced the generation of RPCs which can be used as a source material to produce larger numbers of clinically viable post-mitotic precursors.

Unfortunately staining for transcription factors such as Pax6 and Chx10 requires a permeabilisation step which kills all of the cells. Therefore it is currently not possible to sort and reculture Pax6/Chx10 co-expressing RPCs in order to generate a higher number of photoreceptors.

Low O_2 can be used in synergy with DKK-1, Noggin and IGF-1 to enhance RPCs production 21 days after plating EBs. However, our results suggested that a prolonged culture may be required in order to generate post-mitotic or mature photoreceptors. We have observed differentiation of ganglion and horizontal cells at the same time point in both conditions but failed to detect photoreceptors (Fig 5.12). Despite this, we demonstrated how lowered O_2 could favour development of photoreceptors as a small population of Crx/IRBP expressing cells at 2% O_2 which was absent in 20% O_2 culture (Fig 5.12). It would be interesting to test whether the formation of these cells from RPCs using soluble factors such as RA and taurine (Osakada et al., 2008) can be enhanced by lowering O_2 tension.

5.9 Conclusion

In this chapter, it was revealed that differentiating human pluripotent cells under the atmospheric O_2 tension is a suboptimal condition for RPCs formation and that the differentiation can be enhanced by lowering the O_2 tension near to the

physiological level. The neural inducing protocol used in this particular system appears to act synergistically with the hypoxic regime employed to induce a significant increase in RPCs differentiation efficiency. Working under hypoxic conditions increased retinal differentiation efficiency for iPSCs and hESCs, thus the effect appears to be generic. This suggests that, in addition to the identified factors, microenvironmetal O_2 also plays a pivotal role in generating RPCs from pluripotent stem cells.

6. Concluding remarks and future work

6.1. Summary of result chapters

In this study, the followings were investigated; i) characterization of human pluripotent stem cell lines, ii) validate a previously reported RPC differentiation protocol using two human pluripotent stem cell lines and iii) the effect of lowering O_2 tension on the generation of RPCs.

In chapter 3, we characterized human iPSCs and hESCs. We revealed that cells of both cell lines remained undifferentiated during *in vitro* culture that differentiated into cells representing all three embryonic germ layers when cultured in suspension (see chapter 3). Confirmation of pluripotency is a critical check point prior to generating specific cell types from human pluripotent stem cells. As mentioned in Section 3.2.1, we identified some colonies in the culture that failed to reprogrammed into iPSCs (Fig 3.1A). In order to examine whether these colonies have similar differentiation potentials as fully reprogrammed colonies, Type I and II colonies wereselected and subjected to the same retinal differentiation protocol (Fig 6.1A). The analysis revealed that cells failed to exhibit morphological characteristics observed with fully reprogrammed iPSCs and hESCs during adherent retinal differentiation (Fig 6.1B) and did not generate RPCs co-expressing Pax6 and Chx10 (date not shown). This provides supporting evidence of the importance of

maintaining pluripotency during *in vitro* culture.

Figure 6.1

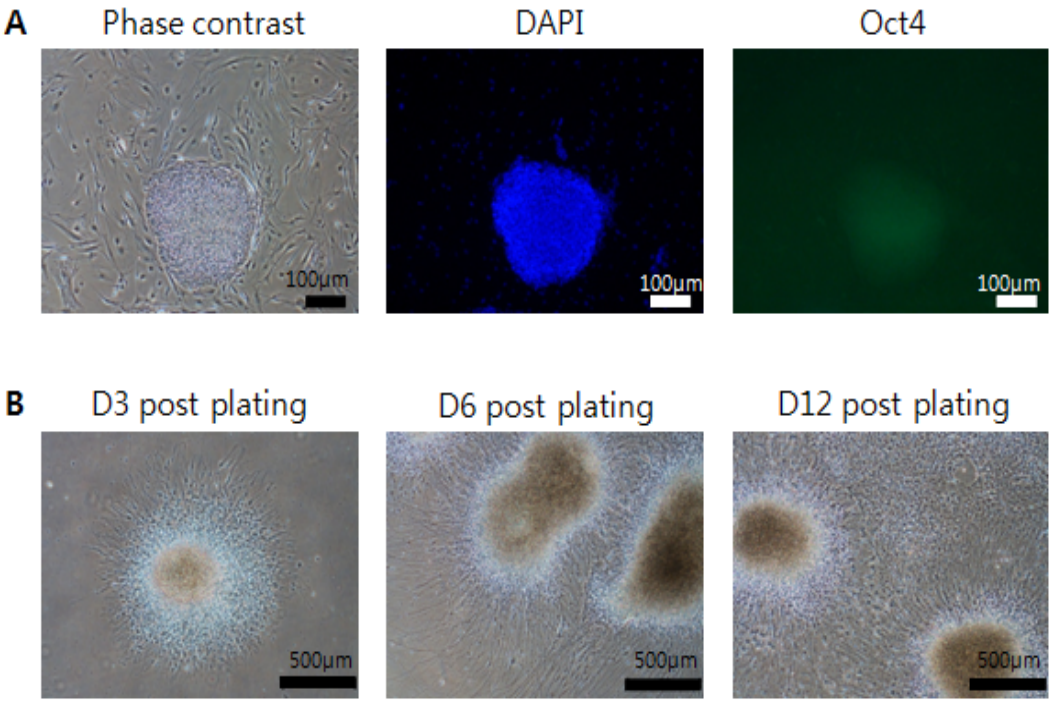


Figure 6.1. Retinal differentiation of Oct4-negative hES-like colonies from MSUH001-iPSCs culture. A) Some colonies exhibiting hESCs-like morphology were Oct4-negative colonies that failed to fully reprogrammed. B) These Oct4-negative colonies were selected and directed to differentiate into RPCs using the Lamba protocol. The morphological analysis during the differentiation period did not show the morphological characteristics observed during iPSCs and hESCs differentiation.

In chapter 4, we investigated whether the Lamba protocol can be repeated using MSUH001-iPS and Shef3-hES cell lines with a comparable efficiency. It was evident that both cell lines differentiated into RPCs co-expressing RPC markers Pax6 and Chx10 but with a varying efficiency. In addition, we discovered that MSUH001-iPS cell line is much favorable in differentiating into RPCs compared to Shef3-hES cell line.

In chapter 5, we examined whether RPC generation could be optimized by lowering O_2 tension during differentiation period. Previous chapter revealed that the percentage of RPCs generated from both cell lines was lower than that reported in a previous study. To enhance RPC differentiation efficiency, we identified O_2 tension as a key variable to achieve the goal and results from the previous chapter were used as control data to quantify any benefits of manipulating O_2 tension. It was revealed that lowering O_2 tension during differentiation significantly enhance the percentage of RPCs co-expressing Pax6 and Chx10 from both cell lines compared to 20% O_2.

6.2. Conclusion

This study has provided promising findings that mimicking physiological O_2 tension enhances generation of RPCs from human pluripotent stem cells. This represents another evidence of a growing trend in stem cell biology that maintaining O_2 at levels found in the developing body is advantageous in differentiating stem cells into specific cell types in the body. The implication of the results is that O_2 tension could be manipulated to increase the production of RPCs from human pluripotent stem cells that could be used to generate clinically viable retinal cells. As mentioned earlier in the chapter, RPCs are capable of generating retinal neurons including photoreceptors, therefore producing larger quantities of RPCs using lowered O_2 tension could subsequently increase the number of photoreceptors for possible transplantation.

6.3. Challenges associated with the research

The biggest challenge for this the research was a "run-to-run" variation of RPC differentiation efficiency. The yield varied from ~10% to as much as ~40% cells co-expressing Pax6 and Chx10 (Fig 6.2). Despite the varying efficiency of RPC differentiation, the effect of lowering O_2 was consistent throughout the repeat runs although the margin was different. It is difficult to pin-point the cause of the efficiency variation however, it was observed that cells differentiate morphologically differently depending on the size and shape of EBs (date not shown). This view is in line with a previously reported study which examined preferential differentiation potential of EBs with varying sizes (Bauwens et al., 2008). Current method used to form EBs involves scraping human pluripotent stem cells colonies which results in morphologically heterogeneous population of EBs. Production of a morphologically uniform and homogenous population of EBs is currently being investigated in our lab and it would be interesting to observe whether the optimum size of EBs for efficiently generation of RPCs could be determined.

Figure 6.2

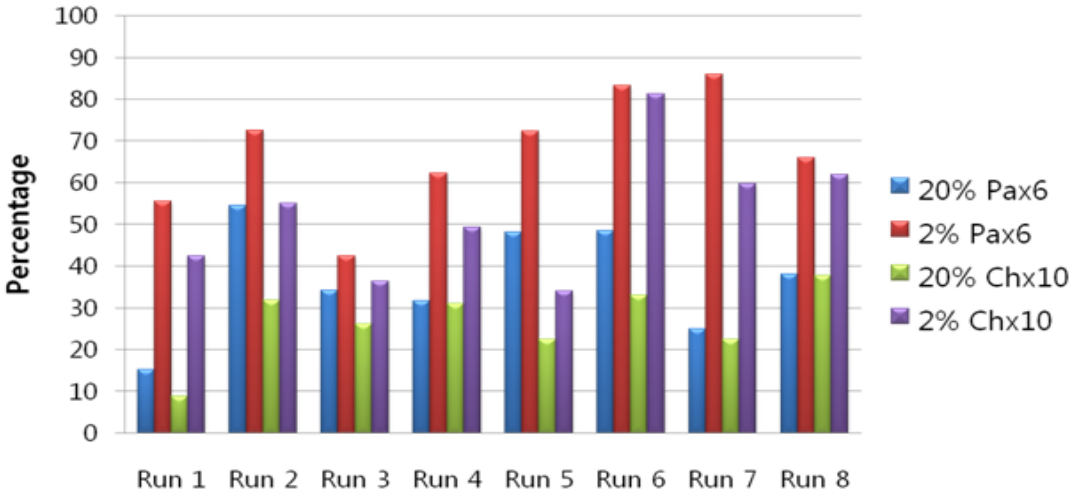


Figure 6.2. Variation of RPC differentiation efficiency over 8 independent runs. The percentage of Pax6 and Chx10 positive cells was inconsistent each time but the effect 2% O_2 tension exerted is consistent in that it increased the generation of RPCs.

6.4. Future works

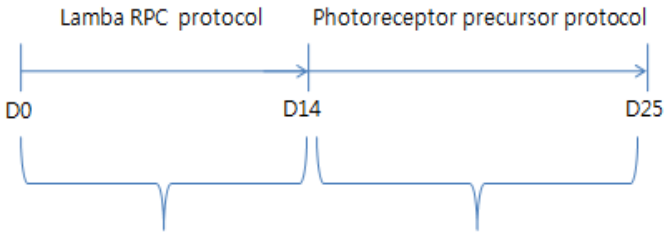
6.4.1. The effect of lowered O_2 on the generation of transplantable retinal cells

It has been well documented in previous studies that RPCs generated from either iPSCs or hESCs have the capabilities to further differentiate into photoreceptor precursor cells (Osakada et al., 2008; Lamba et al., 2010). These post-mitotic cells expressing Nrl, Crx or IRBP have the ability to migrate to the ONL of retina when transplanted subretinally, and develop into mature and functional photoreceptors (McLaren et al., 2006; Lamba et al., 2010). For future work, it would be interesting to observe whether RPCs derived from MSUH001-iPSCs and Shef3-hESCs could also follow the same suit. In order to achieve this, iPSCs and hESCs were induced to differentiate into RPCs in the presence of DKK-1, Noggin, IGF-1 and bFGF up to 14 days after plating EBs. Thereafter, growth factors were withdrawn and factors that are known to induce photoreceptor development such as Taurine and RA were supplemented to the same basal media (Fig. 6.3A). After a several attempts, the preliminary results suggested that possibly supplementing bFGF had exerted detrimental effects on the differentiation of RPCs (Fig. 6.3B). When iPSCs-RPCs were induced to differentiate in the absence of bFGF, small clusters of cells expressed Crx marker (Fig 6.3 B, third attempt and C). In the future, this can be taken further to optimize photoreceptor precursor differentiation for a greater yield of

transplantable Crx^+ cells. This is particularly important as purifying these cells are now possible with newly discovered surface antibodies for photoreceptor precursor cells (Koso et al., 2009; Lakowski et al., 2011). Optimisation strategies for future study may include i) identifying the exact combination of factors and their concentration, ii) exploring the use of different extracellular matrices and iii) using lowered O_2 tension. The use of lowered O_2 tension has been preliminarily test by placing the differentiation culture under 2% O_2 when Taurine and RA were added after withdrawing DKK-1, Noggin, IGF-1 and bFGF. This however, resulted in cell death, demonstrating the timing of 2% O_2 tension switch is equally important and required further investigation (Fig 6.3D).

Figure 6.3

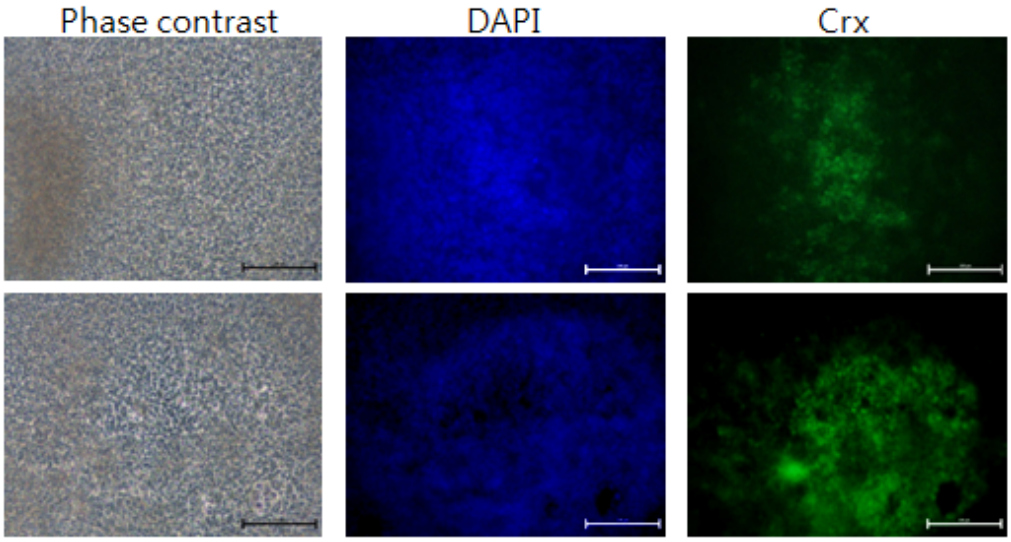
A



B

Attempt	Media composition	Media composition	Outcome
1	DMEM:F12, DKK-1 (10ng/ml), Noggin (10ng/ml), IGF-1(10ng/ml), bFGF(5ng/ml)	DMEM:F12, KOSR(5%), bFGF(100ng/ml), Taurine (100mM), N2 (100U/ml), DAPT (10µm), RA (10µm)	No Crx⁺ cells
2	DMEM:F12, DKK-1 (10ng/ml), Noggin (10ng/ml), IGF-1(10ng/ml), bFGF(5ng/ml)	DMEM:F12, bFGF(20ng/ml), Taurine (100mM), N2 (100U/ml), DAPT (10µm), RA (500nm)	No Crx⁺ cells
3	DMEM:F12, DKK-1 (10ng/ml), Noggin (10ng/ml), IGF-1(10ng/ml), bFGF(5ng/ml)	DMEM:F12, Taurine (100mM), N2 (100U/ml), DAPT (10µm), RA (10µm)	Clusters of Crx⁺ cells (see C)

C



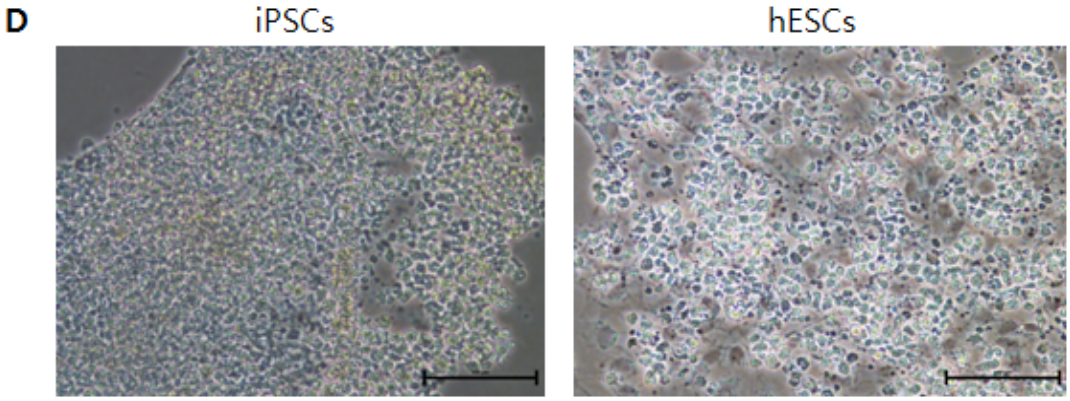


Figure 6.3. Differentiation of photoreceptor precursors from iPSCs- and hESCs- RPC. (A) A schematic of differentiation protocol. The eye field factors (DKK-1, Noggin and IGF-1) are removed 14 days post plating EBs and RA and taurine were supplemented. (B) Different attempts made to generate Crx^{+} cells by using various concentrations of factors that are known to induce photoreceptor development (Taurine, DAPT, RA). (C) Clusters of cells expressing Crx marker after 3rd attempt. (D) Apoptotic cells 2 days after switching the media under 2% O_2. Scale bar: 100µm.

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